Attorney Docket No.: 4394.214-US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FILING UNDER 37 C.F.R. 1.53(b)

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

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Date of Deposit June 3, 1999

Sir:

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b) of

Applicant(s): Svendsen et al.

Title: α-Amylase Mutants

137 pages of specification 13 sheets of formal drawings 21 pages of Sequence Listing

2 sheets of Declaration and Power of Attorney

[x] The filing fee is calculated as follows:

Basic Fee:	$760.00
Total Claims: 2 - 20 = 0 x 18 =	$0
Independent Claims: 2 - 3 = 0 x 78 =	$0
Total Fee:	$760.00

Priority of Danish application serial nos. 1256/95 and 1192/95, filed November 10, 1995 and October 23, 1995, respectively, is claimed under 35 U.S.C. 119. Certified copies thereof were submitted with USSN 08/600,908. A certified copy of 0128/95 filed February 3, 1995 is submitted herewith.

The benefit of application serial no. 08/600,908 filed on February 13, 1996 in the U.S. and PCT/DK96/00057 filed February 5, 1996 in the PCT is claimed under 35 U.S.C. 120.

Address all future communications to Steve T. Zelson, Esq., Novo Nordisk of North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174-6401.

Please charge the required fee, estimated to be $760, to Novo Nordisk of North America, Inc., Deposit Account No. 14-1447. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: June 3, 1999

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Attorney Docket No.: 4394.214-US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Svendsen et al.

Serial No.: To be assigned Group Art Unit: To be assigned

Filed: June 3, 1999 Examiner: To be assigned

For: α-Amylase Mutants

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Before the above-captioned application is taken up for examination, entry of the following amendment is respectfully requested:

IN THE SPECIFICATION:

At page 1, before the first line, insert

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application serial no. 08/600,908 filed February 13, 1996 (now allowed), which is a continuation of PCT/DK96/00057 filed February 5, 1996, and claims priority under 35 U.S.C. 119 of Danish applications 1256/95, 1192/95, and 0128/95 filed November 10, 1995, October 23, 1995, and February 3, 1995, respectively, the contents of which are fully incorporated herein by reference.--

On page 5, line 9, after "WO 95/26397", insert --(SEQ ID NO: 1 of WO 95/26397 is shown in Fig. 8 of the instant application (SEQ ID NO: 12))--.

On page 8, line 28, delete "the Figures" and insert --Figures 1-6--.

On page 43, lines 2 and 9 (both occurrences), delete "SEQ ID NO: 1" and insert --SEQ ID NO: 2--.

On page 65, at the end of line 3, please insert --(SEQ ID NO: 7)--.

On page 65, at the end of line 6, please insert --(SEQ ID NO: 8)--.

On page 65, at the end of line 8, please insert --(SEQ ID NO: 9)--.

At page 65, line 27, after "(Novo Nordisk A/S)", insert --SEQ ID NO:2--.

At page 67, line 21, after "WO 95/26397", insert --(SEQ ID NO:1 of WO 95/26397 is shown in Fig. 8 of the instant application, SEQ ID NO:12)--.

Please delete pages 69-75 (Sequence Listing), insert the substitute Sequence Listing appended herewith (marked as pages 69-89), and re-number the subsequent pages accordingly.

IN THE CLAIMS:

Cancel claims 1-70 without prejudice or disclaimer.

Add new claims 71 and 72 reading as follows:

--71. A method of producing a variant of a parent alpha-amylase having an altered property relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70 % homologous to the sequence of SEQ ID Nos: 2, 4, 6, or 13 when homology is determined by the GAP program (Genetic Computer Group, Version 7.0) using default values for GAP penalties, said method comprising

(a) modelling the parent alpha-amylase on an X-ray crystallographic three-dimensional structure of an alpha-amylase having the sequence of SEQ ID Nos: 2, 4, 6, or 13, or having a sequence at least 70 % homologous to the sequence of SEQ ID Nos: 2, 4, 6, or 13 when homology is determined by the GAP program using default values for GAP penalties, to produce a three-dimensional structure of the parent alpha-amylase;

(b) identifying in the three-dimensional structure obtained in step (a) at least one structural part of the parent wherein an alteration in said structural part is predicted to result in said altered property;

(c) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and

(d) expressing the modified nucleic acid to produce the variant alpha-amylase, wherein the variant has alpha-amylase enzymatic activity and has at least one altered property relative to the parent. --

--72. A method of constructing a variant of a parent alpha-amylase having an altered property relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13 or has a sequence at least 70% homologous to the sequence of SEQ ID Nos: 2, 4, 6, or 13 when homology is determined by the GAP program (Genetic Computer Group, Version 7.0) using default values for GAP penalties, said method comprising

(a) modelling the parent alpha-amylase on an X-ray crystallographic three-dimensional structure of an alpha-amylase having the sequence of SEQ ID Nos: 2, 4, 6, or 13, or having a sequence at least 70% homologous to the sequence of SEQ ID Nos: 2, 4, 6, or 13 when homology is determined by the GAP program using default values for GAP penalties, to produce a three-dimensional structure of the parent alpha-amylase;

(b) comparing the three-dimensional structure obtained in step (a) with a three-dimensional structure of an unrelated alpha-amylase, wherein the unrelated alpha-amylase differs from the parent alpha-amylase in said property;

(c) identifying a structural part of the three-dimensional structure obtained in step (a) which is different from the three-dimensional structure of the unrelated alpha-amylase and which is predicted to be relevant to said property,

(d) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and

(e) expressing the modified nucleic acid to produce the variant alpha-amylase, wherein the variant has alpha-amylase activity and has one or more altered properties as compared to the parent alpha-amylase. --

REMARKS

Entry of this amendment is respectfully requested.

In this response, claims 1-70 are cancelled without prejudice and new claims 71 and 72 are added. Support for the new claims can be found in the specification and original claims. For example, the definition of a parent (Termamyl-like) alpha-amylase can be found in the specification at page 5, lines 4-20. The process of modelling a parent alpha-amylase on a three-dimensional structure is described on page 12, lines 7-24; and on page 62, line 4 - page 63, line 5 (Example 1). Production of a variant alpha-amylase by modification

of a nucleic acid sequence and expression of the modified sequence is disclosed on page 13, lines 12-20. Alpha-amylases unrelated to the parent alpha-amylases as defined in the present claims are described at page 15, lines 11-17. No new matter is added. Accordingly, claims 71 and 72 are pending and at issue.

Sequence Listing

Appended herewith is a request to transfer the Sequence Listing from the parent application to this application.

The content of the attached paper entitled "SEQUENCE LISTING" is identical to the information in the specification as originally filed. No new matter is added.

Rejection of Claims Under 35 U.S.C. § 103(a)

In the parent application, claims similar in scope to present claims 71 and 72 were rejected under 35 U.S.C. § 103(a) as unpatentable over Machius et al., *J. Mol. Biol.* 246:545, 1995, and MacGregor, *J. Prot. Chem.* 7, 399, 1988, further in view of Svendsen et al., WO 94/02597 (see, Office Action of December 8, 1998, in application serial no. 08/600,908, now allowed). The Examiner contended that Machius et al. discloses the structure of calcium-depleted α-amylase from *B. licheniformis*; MacGregor teaches how to predict α-amylase structure; Svendsen et al. teaches recombinant production of α-amylases; and that it would have been been obvious to combine the teachings in these references to achieve the claimed invention. The Examiner also contended that Applicants were only entitled to the priority date of October 23, 1995, for the three-dimensional structure disclosed in the Appendix of the present application. This rejection is respectfully traversed.

Attached herewith is a certified copy of the first priority application, DK 0128/95, which was filed on February 3, 1995. The Examiner's attention is directed to the Appendix (following the figures), showing that the coordinates describing the three-dimensional structure of a *Bacillus* α-amylase were disclosed in this application. It is believed on this basis that Applicants are entitled to the priority date of February 3, 1995 for the presently claimed invention.

On this basis, it is respectfully submitted that Macchius et al., which was published in March, 1995, is not a reference against the present claims.

As discussed with the Examiner in an interview with Applicants' representative and the inventors on May 27, 1999, MacGregor provides almost no useful information regarding the structure of *Bacillus* α-amylases beyond elucidating the central β-barrel structure and the structure of some of the surrounding α-helices. Thus, one of ordinary skill in the art, based on MacGregor, with or without Svensson et al., could have no reasonable expectation of achieving α-amylase variants with predictively altered properties.

Accordingly, it is respectfully submitted that present claims 71 and 72 are free of the prior art.

Furthermore, and as further discussed at the May 27, 1998, interview, Applicants believe that the striking structural homologies between α-amylases belonging to the "Termamyl-like" α-amylase family (i.e., α-amylases having the sequence of SEQ ID Nos: 2, 4, 6, or 13 of the present specification or having a sequence at least 70% homologous to the sequence of SEQ ID Nos: 2, 4, 6, or 13, as required by the present claims) fully support the scope of the present claims.

In view of the above amendments and remarks, it is believed that the claims are in condition for allowance, and a determination to that effect is earnestly solicited.

Respectfully submitted,

Date: June 3, 1999

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Attorney Docket No. 4394.204-US

α-AMYLASE MUTANTS

FIELD OF THE INVENTION

The present invention relates to a novel method of designing α-amylase mutants with predetermined properties, which method is based on the hitherto unknown three-dimensional structure of bacterial α-amylases.

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) constitute a group of enzymes which is capable of hydrolyzing starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides. Almost all α-amylases studied have a few conserved regions with approximately the same length and spacing. One of these regions resembles the Ca^{2+} binding site of calmodulin and the others are thought to be necessary for the active center and/or binding of the substrate.

While the amino acid sequence and thus primary structure of a large number of α-amylases are known, it has proved very difficult to determine the three-dimensional structure of all α-amylases. The three-dimensional structure can be determined by X-ray crystallographic analysis of α-amylase crystals, but it has proven difficult to obtain α-amylase crystals suitable for actually solving the structure.

Until now the three-dimensional structure of only a few α-amylases have been determined at high resolution. These include the structure of the *Aspergillus oryzae* TAKA α-amylase (Swift et al., 1991), the *Aspergillus niger* acid amylase (Brady et al., 1991), the structure of pig pancreatic α-amylase (Qian et al., 1993), and the barley alpha-amylase (Kadziola et al. 1994, Journal of Molecular Biology 239: 104-121, A.Kadziola, Thesis, Dept of Chemistry, U. of Copenhagen, Denmark). Furthermore, the three-dimensional structure of a *Bacillus circulans* cyclodextrin glycosyltransferase (CGTase) is known (Klein et al., 1992) (Lawson et al., 1994). The CGTase catalyzes the same type of reactions as α-amylases and exhibits some structural resemblance with α-amylases.

Furthermore, crystallization and preliminary X-ray studies of *B. subtilis* α-amylases have been described (Chang et al. (1992) and Mizuno et al. (1993)). No final *B. subtilis* structure has been reported. Analogously, the preparation of *B. licheniformis*

α-amylase crystals has been reported (Suzuki et al. (1990), but no subsequent report on X-ray crystallographic analysis or three-dimensional structure are available.

Several research teams have attempted to build three-dimensional structures on the basis of the above known α-amylase structures. For instance, Vihinen et al. (J. Biochem. 107, 267-272, 1990), disclose the modelling (or computer simulation) of a three-dimensional structure of the *Bacillus stearothermophilus* α-amylase on the basis of the TAKA amylase structure. The model was used to investigate hypothetical structural consequences of various site-directed mutations of the *B. stearothermophilus* α-amylase. E.A. MacGregor (1987) predicts the presence of α-helices and ß-barrels in α-amylases from different sources, including barley, pig pancreas and *Bacillus amyloliquefaciens* on the basis of the known structure of the *A. oryzae* TAKA α-amylase and secondary structure predicting algorithms. Furthermore, the possible loops and subsites which may be found to be present in, e.g., the *B. amyloliquefaciens* α-amylase are predicted (based on a comparison with the *A. oryzae* sequence and structure).

A.E. MacGregor (Starch/Stärke 45 (1993), No. 7, p. 232-237) presents a review of the relationship between the structure and activity of α-amylase related enzymes.

Hitherto, no three-dimensional structure has been available for the industrially important *Bacillus* α-amylases (which in the present context are termed "Termamyl-like α-amylases"), including the *B. licheniformis*, the *B. amyloliquefaciens*, and the *B. stearothermophilus* α-amylase.

BRIEF DISCLOSURE OF THE INVENTION

The three-dimensional structure of a Termamyl-like bacterial α-amylase has now been elucidated. On the basis of an analysis of said structure it is possible to identify structural parts or specific amino acid residues which from structural or functional considerations appear to be important for conferring the various properties to the Termamyl-like α-amylases. Furthermore, when comparing the Termamyl-like α-amylase structure with known structures of the fungal and mammalian α-amylases mentioned above, it has been found that some similarities exist between the structures, but also that some striking, and not previously predicted structural differences between the α-amylases exist. The present invention is based on these findings.

Accordingly, in a first aspect the invention relates to a method of constructing a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity and at least one altered property as compared to said parent α-amylase, which method comprises

i) analyzing the structure of the Termamyl-like α-amylase with a view to identifying at least one amino acid residue or at least one structural part of the Termamyl-like α-amylase structure, which amino acid residue or structural part is believed to be of relevance for altering said property of the parent Termamyl-like α-amylase (as evaluated on the basis of structural or functional considerations),

ii) constructing a Termamyl-like α-amylase variant, which as compared to the parent Termamyl-like α-amylase, has been modified in the amino acid residue or structural part identified in i) so as to alter said property, and

iii) testing the resulting Termamyl-like α-amylase variant for said property.

In a second aspect the present invention relates to a method of constructing a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity and one or more altered properties as compared to said parent α-amylase, which method comprises

i) comparing the three-dimensional structure of the Termamyl-like α-amylase with the structure of a non-Termamyl-like α-amylase,

ii) identifying a part of the Termamyl-like α-amylase structure which is different from the non-Termamyl-like α-amylase structure, and

iii) modifying the part of the Termamyl-like α-amylase identified in ii) whereby a Termamyl-like α-amylase variant is obtained, one or more properties of which differ from the parent Termamyl-like α-amylase.

In a third aspect the invention relates to a method of constructing a variant of a parent non-Termamyl-like α-amylase, which variant has α-amylase activity and one or more altered properties as compared to said parent α-amylase, which method comprises

i) comparing the three-dimensional structure of the non-Termamyl-like α-amylase with the structure of a Termamyl-like α-amylase,

ii) identifying a part of the non-Termamyl-like α-amylase structure which is different from the Termamyl-like α-amylase structure, and

iii) modifying the part of the non-Termamyl-like α-amylase identified in ii) whereby a non-Termamyl-like α-amylase variant is obtained, one or more properties of which differ from the parent Termamyl-like α-amylase.

The property which may be altered by the above methods of the present invention may, e.g., be substrate specificity, substrate binding, substrate cleavage pattern, temperature stability, pH dependent activity, pH dependent stability (especially increased stability at low (e.g. pH<6, in particular pH<5) or high (e.g. pH>9) pH values), stability towards oxidation, Ca^{2+}-dependency, specific activity, and other properties of interest. For instance, the alteration may result in a variant which, as compared to the parent Termamyl-like α-amylase, has an increased specific activity at a given pH and/or an altered substrate specificity.

In still further aspects the invention relates to variants of a Termamyl-like α-amylase, DNA encoding such variants and methods of preparing the variants. Finally, the invention relates to the use of the variants for various industrial purposes.

DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α-amylase

It is well known that a number of alpha-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 (commercially available as Termamyl®) has been found to be about 89% homologous with the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID No. 4 and about 79% homologous with the *B. stearothermophilus* α-amylase comprising the amino acid sequence shown in SEQ ID No. 6. Further homologous α-amylases include an α-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α-amylase described by Tsukamoto et al., 1988, Biochemical and Biophysical Research Communications, Vol. 151, No. 1. Still other homologous α-amylases include the α-amylase produced by the *B. licheniformis* described in EP 252 666 (ATCC 27811), and the α-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α-amylases are Optitherm® and Takatherm® (available from Solvay), Maxamyl® (available from Gist-brocades/Genencor), Spezym AA® (available from Genencor), and Keistase® (available from Daiwa).

Because of the substantial homology found between these α-amylases, they are considered to belong to the same class of α-amylases, namely the class of "Termamyl-like α-amylases".

Accordingly, in the present context, the term "Termamyl-like α-amylase" is intended to indicate an α-amylase which, on the amino acid level, exhibits a substantial homology to Termamyl®, i.e. the *B. licheniformis* α-amylase SEQ ID NO 2. In other words, a Termamyl-like α-amylase is an α-amylase, which has the amino acid sequence shown in SEQ ID No. 2, 4 or 6 herein, or the amino acid sequence shown in SEQ ID NO 1 or 2 of WO 95/26397 or in Tsukamoto et al., 1988, or i) which displays at least 60%, such as at least 70%, e.g. at least 75%, or at least 80%, e.g. at least 85%, at least 90% or at least 95% homology with at least one of said amino acid sequences and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α-amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above specified α-amylases which are apparent from SEQ ID Nos. 1, 3 and 5 of the present application, and SEQ ID NO 4 and 5 of WO 95/26397, respectively.

In connection with property i) the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP progamme from the GCG package version 7.3 (June 1993) using default values for GAP penalties (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711).

Property ii) of the α-amylase, i.e. the immunological cross reactivity, may be assayed using an antibody raised against or reactive with at least one epitope of the relevant Termamyl-like α-amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., 1989. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g. as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α-amylases having the amino acid sequences SEQ ID Nos. 2, 4 and 6, respectively, has been found.

The oligonucleotide probe used in the characterization of the Termamyl-like α-amylase in accordance with property iii) above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α-amylase in question. Suitable

conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1 h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 μg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 μM ATP for 18 h at ~40°C, or other methods described by e.g. Sambrook et al., 1989.

In the present context, "derived from" is intended not only to indicate an α-amylase produced or producible by a strain of the organism in question, but also an α-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α-amylase in question. The term is also intended to indicate that the parent α-amylase may be a variant of a naturally occurring α-amylase, i.e. a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α-amylase.

<u>Parent hybrid α-amylases</u>

The parent α-amylase (being a Termamyl-like or non-Termamyl-like α-amylase) may be a hybrid α-amylase, i.e. an α-amylase which comprises a combination of partial amino acid sequences derived from at least two α-amylases.

The parent hybrid α-amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α-amylase family. In this case, the hybrid α-amylase is typically composed of at least one part of a Termamyl-like α-amylase and part(s) of one or more other α-amylases selected from Termamyl-like α-amylases or non-Termamyl-like α-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α-amylase may comprise a combination of at least two Termamyl-like α-amylases, or of at least one Termamyl-like and at least one non-Termamyl-like bacterial α-amylase, or of at least one Termamyl-like and at least one fungal α-amylase. For instance, the parent α-amylase comprises a C-terminal part of an α-amylase derived from a strain of *B. licheniformis* and a N-terminal part of an α-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α-amylase comprises at least 430 amino acid residues of the C-terminal part of the *B.*

licheniformis α-amylase, and may, e.g. comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* α-amylase having the amino acid sequence shown in SEQ ID No. 4 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2, or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α-amylase having the amino acid sequence shown in SEQ ID No. 6 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2.

Analogously, the parent hybrid α-amylase may belong to a non-Termamyl-like α-amylase family, e.g. the Fungamyl-like α-amylase family. In that case the hybrid may comprise at least one part of an α-amylase belonging to the non-Termamyl-like α-amylase family in combination with one or more parts derived from other α-amylases.

<u>The three-dimensional Termamyl-like α-amylase structure</u>

The Termamyl-like α-amylase which was used to elucidate the three-dimensional structure forming the basis for the present invention consists of the 300 N-terminal amino acids of the *B. amyloliquefaciens* α-amylase (with the amino acid sequence shown in SEQ ID No. 4) and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α-amylase with the amino acid sequence SEQ ID No. 2. The bacterial α-amylase belongs to the "Termamyl-like α-amylase family" and the present structure is believed to be representative for the structure of any Termamyl-like α-amylase.

The structure of the α-amylase was solved in accordance with the principle for X-ray crystallographic methods given in "X-Ray Structure Determination", Stout, G.K. and Jensen, L.H., John Wiley & Sons, inc. NY, 1989. The structural coordinates for the solved crystal structure of the α-amylase at 2.2 Å resolution using the isomorphous replacement method are given in a standard PDB format (Brookhaven Protein Data Base) in Appendix 1. It is to be understood that Appendix 1 forms part of the present application.

Amino acid residues of the enzyme are identified by three-letter amino acid code (capitalized letters).

The α-amylase structure is made up of three globular domains ordered A, B, and C with respect to sequence, which lie approximately along a line in the order B, A, C.

The domains can be defined as being residues 1-103 and 206-395 for domain A, residues 104-205 for domain B, and residues 396-483 for domain C, the numbers referring to the *B. licheniformis* α-amylase. This gives rise to an elongated molecule, the longest axis being about 85 Å. The widest point perpendicular to this axis is approximately 50 Å and spans the central A domain. The active site residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) are D323, D231 and E261.

Domain A

Domain A is the largest domain and contains the active site (comprised of a cluster of three amino acid residues placed at the bottom of a deep cleft in the enzyme's surface). Domain A of all known α-amylase structures have the same overall fold, viz. the (beta/alpha)8 barrel with 8 central beta strands (number 1-8) and 8 flanking α-helices. The β-barrel is defined by McGregor *op. cit.* The C-terminal end of Beta strand 1 is connected to helix 1 by a loop denoted loop 1 and an identical pattern is found for the other loops. These loops show some variation in size and some can be quite extensive.

The 8 central Beta-strands in the (beta/alpha)8 barrel superimpose well between the various known α-amylase structures, and this part of the structure, including the close surroundings of the active site located at the c-terminal end of the beta-strands, show high similarity between the different amylases.

The loops connecting beta-strands and alpha helices display high variations between alpha amylases. These loops constitute the structural context of the active site and the majority of the contacts to the substrate is found among residues located in these loops. Such important characteristics as substrate specificity, substrate binding, pH/activity profile, starch cleavage pattern are determined by the amino acids and the positions of same in these loops.

The substantial differences between the Fungamyl-like α-amylase structure and the structure of the Termamyl-like α-amylase disclosed herein which are found in loops 1, 2, 3, and 8 are visualized in the Figures.

Domain B

The Termamyl-like α-amylase structure has been found to comprise a special domain structure in the A domain's loop3, also called domain B. The structure of the

Termamyl-like α-amylase B domain has never been seen before in any of the known α-amylase or (β/alpha)8-barrel proteins.

The domain B structure is a very compact domain having a very high number of charged residues. The B domain arises as an extension of the loop between strand 3 and helix 3 of domain A (shown in Fig. 7) and contains a 5 stranded antiparallel β-sheet structure containing at least one long loop structure and having the connectivity -1, +3, -1X, +2 (Richardson, 1981, Adv. Protein Chem. 34, 167-339).

The first four strands of the B domain form two hairpin loops which twist around each other like a pair of crossed fingers (right-hand twist). The main chain folds into a ß-strand which connects two small ß-sheet structures. After making one turn in one sheet it folds back and makes up a two stranded sheet in contact with domain A and an internal hole in the α-amylase structure. Then the main chain folds up to a small sheet structure nearly perpendicular to the first two sheets. Before entering the helix 3 on top of the ß-strand 3, the approximately 24 last amino acids in domain B form two calcium binding sites in the contact region to domain A.

Domain B is connected with domain A by two peptide stretches, which divide the domain-domain contact areas into two. Domain B is in contact with Domain A by a calcium binding region and an internally buried hole containing waters. Many types of molecular contacts are present. Ionic interacting between acid and basic amino acids are possible, these interactions are very important for the general stability at high pH and for keeping the Calcium binding sites intact.

Domain C

Domain C is the C-terminal part of the protein consisting of amino acids 394-483. Domain C is composed entirely of β-strands which forms a single 8-stranded sheet structure, which folds back on itself, and thus may be described as a β-sandwich structure. The connectivity is +1,+1, +5, -3, +1, +1, -3 although strands 6 and 7 are only loosely connected. One part of the β-sheet forms the interface to domain A.

Ca-binding and Na-binding sites

The structure of the Termamyl-like α-amylase is remarkable in that it exhibits four calcium-binding sites and one sodium-binding site. In other words four calcium ions

and one sodium ion are found to be present in the structure, although one of the calcium ions displays very weak coordination. Two of the calcium ions form part of a linear cluster of three ions, the central ion being attributed to sodium, which lie at the junction of the A and B domains.

The coordinating residues for the calcium ions between the A and B domain are as follows (using the Pdb file nomenclature for amino acid residues and atoms in the Pdb file found in Appendix 1 herein): For the calcium ion nearest to the active site (IUM 502 in the pdb file), the backbone carbonyls from His235 and Asp194, the sidechain atom OD1 from residues Asp194, Asn102 and Asp200, and one water molecule WAT X3 (atom OW7). For the sodium ion (IUM 505), the binding site includes atom OD2 from Asp194, Asp200, Asp183 and Asp159, and a backbone carbonyl from Val201. The coordinates for the other calcium ion between domain A and B are (IUM 501) : atom OD2 from Asp204 and Asp159, backbone carbonyl from Asp183 and Ala181, atom OD1 from Asp202, and one water molecule WAT X7 (atom OW7).

One calcium ion is located between the A and C domain, another is located in the C domain. The first mentioned calcium ion, which is also the one best coordinated (IUM 503) includes a carbonyl backbone from Gly300, Tyr302 and His406, atom OD2/OD1 from Asp430, atom OD1 from Asp407, and one water molecule WAT X6 (atom OW7). The other and very weakly coordinated calcium site (IUM 504) comprises 4 water molecules WAT X21 (atom OW8), X6 (atom OW6), X9 (atom OW0) and X28 (atom OW8), OE1/OE2 from Glu447 and OD1 from Asn444.

Substrate-binding site

Without being limited to any theory it is presently believed that favorable interactions between a substrate molecule and the enzyme (such as hydrogen bonds and/or strong electrostatic interaction) are found within a sphere of 4 Å of the substrate, when bound to the enzyme. The following residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 are contemplated to be within a distance of 4 Å of the substrate and thus believed to be involved in interactions with the substrate:
Trp13, Tyr14, Asn17, Asp18, Ser50, Gln51, Ala52, Asp53, Val54, Gly55, Tyr56, Lys70, Arg74, Lys76, Val102, His105, Gly107, Gly108, Ala109, Trp138, Thr163, Asp164, Trp165,

Asn172, Glu189, Tyr193, Leu196, Met197, Tyr198, Ala199, Arg229, Asp231, Ala232, Lys234, His235, Glu261, Trp263, His327, Asp328, Gln333, Ser334, and Leu335.

The amino acid residues of another Termamyl-like α-amylase, which are contemplated to be within a distance of 4Å of the substrate, may easily be identified by aligning the amino acid sequence SEQ ID NO 2 with that of the other Termamyl-like α-amylase and thereby identifying the positions equivalent to those identified above.

Generality of structure

Because of the high homology between the various Termamyl-like α-amylases, the solved structure defined by the coordinates of Appendix 1 is believed to be representative for the structure of all Termamyl-like α-amylases. A model structure of other Termamyl-like α-amylases may easily be built on the basis of the coordinates given in Appendix 1 adapted to the α-amylase in question by use of an alignment between the respective amino acid sequences. The creation of a model structure is exemplified in Example 1.

The above identified structurally characteristic parts of the Termamyl-like α-amylase structure (Ca-binding site, substrate binding site, loops, etc.) may easily be identified in other Termamyl-like α-amylases on the basis of a model (or solved) structure of the relevant Termamyl-like α-amylase or simply on the basis of an alignment between the amino acid sequence of the Termamyl-like α-amylase in question with that of the *B. licheniformis* α-amylase used herein for identifying the amino acid residues of the respective structural elements.

Furthermore, in connection with Termamyl-like variants of the invention, which are defined by modification of specific amino acid residues of a specific Termamyl-like α-amylase, it will be understood that variants of another Termamyl-like α-amylase modified in an equivalent position (as determined from the best possible amino acid sequence alignment between the respective sequences) are intended to be covered as well. Thus, irrespective of whether an amino acid residue is identified herein for the purpose of defining a structural part of a given α-amylase or used for identifying a variant of the α-amylase, this amino acid residue shall be considered as representing the equivalent amino acid residue of any other Termamyl-like α-amylase.

Methods of the invention for design of novel α-amylase variants

In the methods according to the first, second and third aspects of the invention the terms "structure of a Termamyl-like α-amylase" and "Termamyl-like α-amylase structure" are intended to indicate the solved structure defined by the coordinates presented in Appendix 1 or a model structure of a given Termamyl-like α-amylase (such as the *B. licheniformis* α-amylase) built on the basis of the solved structure.

In most cases the parent Termamyl-like α-amylase to be modified in accordance with the present invention is different from the α-amylase which was actually used for solving the structure (Appendix 1). This means that the amino acid residue(s) or structural part(s) identified in the solved structure (Appendix 1) in step i) of the method according to the first, second or third aspect of the invention must be translated into the corresponding amino acid residue(s) or structural part(s) of the parent Termamyl-like α-amylase in question. The "translation" is conveniently performed on the basis of an amino acid sequence alignment between the amino acid sequence of the Termamyl-like α-amylase used for solving the structure and the amino acid sequence of the parent Termamyl-like α-amylase in question.

The analysis or comparison performed in step i) of the method according to the first, second and third aspect, respectively, of the invention may be performed by use of any suitable computer program capable of analyzing and/or comparing protein structures, e.g. the computer program Insight, available from Biosym Technologies, Inc. For instance, the basic principle of structure comparison is that the three-dimensional structures to be compared are superimposed on the basis of an alignment of secondary structure elements (such as the central 8 β-strands in the barrel) and the parts differing between the structures can subsequently easily be identified from the superimposed structure.

The structural part which is identified in step i) of the methods of the first, second and third aspects of the invention may be composed of one amino acid residue. However, normally the structural part comprises more than one amino acid residue, typically constituting one of the above parts of the Termamyl-like α-amylase structure such as one of the A, B, or C domains, an interface between any of these domains, a calcium binding site, a loop structure, the substrate binding site, or the like.

In the present context the term "structural or functional considerations" is intended to indicate that modifications are made on the basis of an analysis of the relevant

structure or structural part and its contemplated impact on the function of the enzyme. Thus, an analysis of the structures of the various α-amylases, which until now has been elucidated, optionally in combination with an analysis of the functional differences between these α-amylases, may be used for assigning certain properties of the α-amylases to certain parts of the α-amylase structure or to contemplate such relationship. For instance, differences in the pattern or structure of loops surrounding the active site may result in differences in access to the active site of the substrate and thus differences in substrate specificity and/or cleavage pattern. Furthermore, parts of a Termamyl-like α-amylase involved in or contemplated to be involved in substrate binding (and thus e.g. specificity/cleavage pattern), calcium or sodium ion binding (e.g. of importance for the Calcium-dependency of the enzyme), and the like has been identified (*vide infra*).

The modification of an amino acid residue or structural part is typically accomplished by suitable modifications of a DNA sequence encoding the parent enzyme in question. The term "modified" as used in step ii) in the method according to the first aspect of the invention is intended to have the following meaning: When used in relation to an amino acid residue the term is intended to mean replacement of the amino acid residue in question with another amino acid residue. When used in relation to a structural part, the term is intended to mean replacement of one or more amino acid residues of said structural part, addition of one or more amino acid residues to said part, or deletion of one or more amino acid residues of said structural part.

The construction of the variant of interest is accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth. This is described in detail further below.

First aspect of the invention

In a preferred embodiment of the method according to the first aspect of the invention the property of the parent enzyme to be modified is selected from calcium dependency, substrate binding, cleavage pattern, pH dependent activity and the like. Specific examples of how to change these properties of a parent Termamyl-like α-amylase are given further below.

In another preferred embodiment the parent Termamyl-like α-amylase to be modified is a *B. licheniformis* α-amylase.

Second and third aspects of the invention

One important advantage of the methods according to the second and third aspects of the present invention is that it is possible to adapt the structure (or a structural part) of a Termamyl-like α-amylase to the structure (or structural part) of a non-Termamyl-like α-amylase and *vide versa*. For instance, having identified a loop structure of the non-Termamyl-like α-amylase which is believed to be responsible for or contributing to a particular property of the non-Termamyl-like α-amylase it is possible to replace the corresponding structure of the Termamyl-like α-amylase with said non-Termamyl-like α-amylase structure - or if no corresponding structure exists in the Termamyl-like α-amylase - to insert the structure into the Termamyl-like α-amylase in such a manner that the resulting variant Termamyl-like α-amylase, as far as the relevant part is concerned, resembles the corresponding part of the non-Termamyl-like α-amylase. When two or more parts of the structure of the parent Termamyl-like α-amylase are modified so as to resemble the corresponding parts of the non-Termamyl-like α-amylase it is possible to increase the resemblance to the non-Termamyl-like α-amylase of the Termamyl-like α-amylase variant and thus to alter the properties of said variant in the direction of those of said non-Termamyl-like α-amylase. Loop modifications are discussed in much further detail further below.

Typically, the modification to be performed in step iii) of the method according to the second aspect of the invention is accomplished by deleting one or more amino acid residues of the part of the Termamyl-like α-amylase to be modified so as to adapt the structure of said part of the parent α-amylase to the corresponding part of the non-Termamyl-like α-amylase; by replacing one or more amino acid residues of the part of the Termamyl-like α-amylase to be modified with the amino acid residues occupying corresponding positions in the non-Termamyl-like α-amylase; or by insertion of one or more amino acid residues present in the non-Termamyl-like α-amylase into a corresponding position in the Termamyl-like α-amylase. For the method according to the third aspect the modification is to be understood analogously, performed on the non-Termamyl-like parent α-amylase rather than the Termamyl-like α-amylase.

In step ii) of the method according to the second or third aspect of the invention the part of the structure to be identified is preferably one which in the folded enzyme is believed to be in contact with the substrate (cf. the disclosure above in the section entitled "Substrate-binding site) or involved in substrate specificity and/or cleavage pattern, and/or one which is in contact with one of the calcium or sodium ions and/or one, which is contributing to the pH or temperature profile of the enzyme, or one which otherwise, from structural or functional considerations, is contemplated to be responsible for differences in one or more properties of the Termamyl-like and non-Termamyl-like α-amylase.

Non-Termamyl-like α-amylase

The non-Termamyl-like α-amylase with which the comparison is made in step i) of the method of the second aspect of the invention and which is the parent α-amylase in the method of the third aspect of the invention, may be any α-amylase, which does not belong to the family of Termamyl-like α-amylases (as defined above) and, which as a consequence thereof, has a different three-dimensional structure. Furthermore, the non-Termamyl-like α-amylase should be one which has, at the time that the method is performed, an elucidated or contemplated three-dimensional structure.

The non-Termamyl-like α-amylase may, e.g., be a fungal α-amylase, a mammalian or a plant α-amylase or a bacterial α-amylase (different from a Termamyl-like α-amylase). Specific examples of such α-amylases include the *Aspergillus oryzae* TAKA α-amylase, the *A. niger* acid α-amylase, the *Bacillus subtilis* α-amylase, the porcine pancreatic α-amylase and a barley α-amylase. All of these α-amylases have elucidated structures which are clearly different from the structure of the Termamyl-like α-amylase shown herein.

The fungal α-amylases mentioned above, i.e. derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α-amylases. In the present disclosure, this family is termed "Fungamyl-like α-amylase" and intends to indicate an α-amylase which exhibits a high homology, i.e. more than 70%, such as 80% homologous (as defined herein) to the fungal α-amylase derived from *Aspergillus oryzae,* commercially available as Fungamyl®, and the *A. niger* α-amylase.

From the enclosed illustrations of the α-amylase structure of a Termamyl-like α-amylase and a comparison of said structure with the structure of a Fungamyl-like α-

amylase it is evident that major differences exist between the two structures. In the method of the invention it is of particular interest to modify parts of the parent Termamyl-like α-amylase, which belong to a region with large differences to the Fungamyl-like α-amylase. In particular, it is of interest to modify the parent Termamyl-like α-amylase in one or more of the following loops: loop 1, loop 2, loop 3 and/or loop 8 of the parent α-amylase.

In the method of the third aspect of the invention it is of particular interest to modify loop 1, loop 2, loop 3 and/or loop 8 of the parent non-Termamyl-like α-amylase to a closer resemblance to the similar loops of a Termamyl-like α-amylase, such as Termamyl.

In the following specific types of variants are described which have been designed by use of the method of the invention.

Loop modifications

In order to change the substrate specificity of the parent α-amylase to be modified it is relevant to consider loop modifications. For instance changing one or more of the loop structures of the Termamyl-like α-amylase into a closer resemblance with the corresponding loop structure(s) of a non-Termamyl-like α-amylase (such as a Fungamyl-like α-amylase) it is contemplated that it is possible to change the substrate specificity in the direction of that of the non-Termamyl α-amylase. In the following different types of loop modifications of interest are listed. It will be understood that the variants may have other changed properties in addition to the modified substrate specificity. It will be understood that the following modifications identified for a specific Termamyl-like α-amylase are intended to include corresponding modifications in other equivalent positions of other Termamyl-like α-amylases. Furthermore, it will be understood that, normally, the loop modification will comprise replacement of an entire loop structure or a substantial part thereof in, e.g., the Termamyl-like α-amylase, with the corresponding loop structure (or substantial part thereof) in a non-Termamyl-like α-amylase.

Loop2 modifications

In one embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant, at least one amino acid residue of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 44-57 of the amino acid sequence of SEQ ID No. 4, i.e. loop 2, has been deleted or replaced with one or more

amino acid residues which is/are present in a fragment corresponding to the amino acid fragment 66-84 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

The amino acid sequence shown in SEQ ID No. 10 is the amino acid sequence of the *A. oryzae* α-amylase, i.e. a Fungamyl-like α-amylase. It will be understood that amino acid residues or fragments found in corresponding positions in other α-amylases, in particular Fungamyl-like α-amylases, may be used as a template for the construction of the variant according to the invention. The corresponding part in other homologous α-amylases may easily be identified on the basis of a comparison of the amino acid sequences and/or three-dimensional structures of the respective α-amylases.

For instance, the variant may be one, which, when the amino acid sequence of the variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80% such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein X is the amino acid residue occupying position 44, 45, 46, 47 or 48 of SEQ ID No. 4, Y is the amino acid residue occupying position 51, 52, 53, 54, 55, 56 or 57 of SEQ ID No. 4, Z is the amino acid residue occupying position 66, 67, 68, 69 or 70 of SEQ ID No. 10, and V is the amino acid residue occupying position 78, 79, 80, 81, 82, 83 or 84 of SEQ ID No. 10.

In other words, the variant may be one in which an amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 44-57 of SEQ ID No. 4, has been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 66-84 of the amino acid sequence shown in SEQ ID No. 10, in X, Y, Z and V have the meaning indicated above.

A specific example of a variant according to this embodiment is a variant of a parent Termamyl-like α-amylase, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 48-51 of SEQ ID No. 4, has been replaced with an amino acid fragment corresponding to amino acid residues 70-78 of the amino acid sequence shown in SEQ ID No. 10.

Loop 3 modifications - limited alteration

In another embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to the amino acid fragment 195-202 of the amino acid sequence of SEQ ID No. 4, has been deleted or replaced with one or more of the amino acid residues which is/are present in an amino acid fragment corresponding to the amino acid fragment 165-177 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one in which an amino acid fragment X-Y of the parent α-amylase which corresponds to or is within the amino acid fragment 195-202 of SEQ ID No. 4, has been replaced by an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 165-177 of the amino acid sequence shown in SEQ ID No. 10, in which X is an amino acid residue corresponding to the amino acid occupying position 195 or 196 of SEQ ID No. 4, Y is an amino acid residue corresponding to the amino acid occupying position 198, 199, 200, 201, or 202 of SEQ ID No. 4, Z is an amino acid residue corresponding to the amino acid occupying position 165 or 166 of SEQ ID No. 10, and V is an amino acid residue corresponding to the amino acid occupying position 173, 174, 175, 176 or 177 of SEQ ID No. 10.

Expressed in another manner, the variant according to this aspect may be one, which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent Termamyl-like α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, the meaning of X, Y, Z and V being as identified above.

A specific example of a variant according to this embodiment is a variant of a parent Termamyl-like α-amylase, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 196-198 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 166-173 of the amino acid sequence shown in SEQ ID No. 10.

Loop 3 modifications - complete domain B

In a further embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 117-185 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to the amino acid fragment 98-210 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one, in which an amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 117-185 of SEQ ID No. 4, has been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 98-210 of the amino acid sequence shown in SEQ ID No. 10, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 4, Y is an amino acid residue corresponding to the amino acid occupying position 181, 182, 183, 184 or 185 of SEQ ID No. 4, Z is an amino acid residue corresponding to the amino acid occupying position 98, 99, 100, 101, 102 of SEQ ID No. 10, and V is an amino acid residue corresponding to the amino acid occupying position 206, 207, 208, 209 or 210 of SEQ ID No. 10.

A specific example of a variant according to this embodiment is a variant of a parent α-amylase, in which an amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-181 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 102-206 of the amino acid sequence shown in SEQ ID No. 10.

In another embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 117-181 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to the amino acid fragment to 98-206 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added

using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one, in which the amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 117-177 if SEQ ID No. 4, has/have been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 98-202 of the amino acid sequence shown in SEQ ID No. 10, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 4, Y is an amino acid residue corresponding to the amino acid occupying position 174, 175, 176 or 177 of SEQ ID No. 4, Z is an amino acid residue corresponding to the amino acid occupying position 98, 99, 100, 101, 102 of SEQ ID No. 10, and V is an amino acid residue corresponding to the amino acid occupying position 199, 200, 201 or 202 of SEQ ID No. 10.

A specific example of a variant according to this embodiment of the invention is a variant, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-174 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 102-199 of the amino acid sequence shown in SEQ ID No. 10.

Loop 1 modifications - minimal addition

In a further embodiment the present invention relates to a variant of a parent Termamyl-like α-amylase, in which variant at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to the amino acid fragment 12-19 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment which corresponds to the amino acid fragment 28-42 of SEQ ID No. 10, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one, in which the amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 12-19 of SEQ ID No. 4, has/have been replaced with an amino acid fragment Z-V, which

corresponds to or is within the amino acid fragment 28-42 of the amino acid sequence shown in SEQ ID No. 10, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 12, 13 or 14 of SEQ ID No. 4, Y is an amino acid residue corresponding to the amino acid occupying position 15, 16, 17, 18 or 19 of SEQ ID No. 4, Z is an amino acid residue corresponding to the amino acid occupying position 28, 29, 30 , 31 or 32 of SEQ ID No. 10, and V is an amino acid residue corresponding to the amino acid occupying position 38, 39, 40, 41 or 42 of SEQ ID No. 10.

A specific example of a variant according to this aspect of the invention is a variant, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 14-15 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 32-38 of the amino acid sequence shown in SEQ ID No. 10.

Loop 1 modifications - complete loop

In a further embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant at least one of the amino acid residues of the parent α-amylase, which is present in a fragment corresponding to amino acid residues 7-23 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 13-45 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one, in which the amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 7-23 of SEQ ID No. 4, has/have been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 13-45 of the amino acid sequence shown in SEQ ID No. 10, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 7 or 8 of SEQ ID No. 4, Y is an amino acid residue corresponding to the amino acid occupying position 18, 19, 20, 21, 22 or 23 of SEQ ID No. 4, Z is an amino acid residue corresponding to the amino acid occupying position 13 or 14 of SEQ ID No. 10,

and V is an amino acid residue corresponding to the amino acid occupying position 40, 41, 42, 43, 44 or 45 of SEQ ID No. 10.

A specific variant according to this embodiment is one, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 8-18 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 14-40 of the amino acid sequence shown in SEQ ID No. 10.

Loop 8 modifications

In a further embodiment the invention relates to a variant of a parent Termamyl-like α-amylase, in which variant at least one of the amino acid residues of the parent α-amylase, which is present in a fragment corresponding to amino acid residues 322-346 of the amino acid sequence of SEQ ID No. 2, has/have been deleted or replaced with one or more amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 291-313 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

For instance, the variant may be one, in which the amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 322-346 of SEQ ID No. 2, has/have been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 291-313 of the amino acid sequence shown in SEQ ID No. 10, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 322, 323, 324 or 325 of SEQ ID No. 2, Y is an amino acid residue corresponding to the amino acid occupying position 343, 344, 345 or 346 of SEQ ID No. 2, Z is an amino acid residue corresponding to the amino acid occupying position 291, 292, 293 or 294 of SEQ ID No. 10, and V is an amino acid residue corresponding to the amino acid occupying position 310, 311, 312 or 313 of SEQ ID No. 10.

A specific variant according to this aspect of the invention is one, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 325-345 of SEQ D No. 2, has been replaced with the amino acid fragment corresponding to amino acid residues 294-313 of the amino acid sequence shown in SEQ ID No. 10.

Ca^{2+} dependency

It is highly desirable to be able to decrease the Ca^{2+} dependency of a Termamyl-like α-amylase. Accordingly, in a further aspect the invention relates to a variant of a parent Termamyl-like α-amylase, which exhibits α-amylase activity and which has a decreased Ca^{2+} dependency as compared to the parent α-amylase. The decreased Ca^{2+} dependency has the functional result that the variant exhibits a satisfactory amylolytic activity in the presence of a lower concentration of calcium ion in the extraneous medium than is necessary for the parent enzyme and, for example, therefore is less sensitive than the parent to calcium ion-depleting conditions such as those obtained in media containing calcium-complexing agents (such as certain detergent builders).

The decreased Ca^{2+} dependency of the variant of the invention may advantageously be achieved by increasing the Ca^{2+} binding affinity of the parent Termamyl-like α-amylase, in other words the stronger the Ca^{2+} binding of the enzyme, the lower is the Ca^{2+} dependency.

It is presently believed that amino acid residues located within 10 Å from a sodium or calcium ion are involved in or are of importance for the Ca^{2+} binding capability of the enzyme.

Accordingly, the variant according to this aspect of the invention is preferably one, which has been modified in one or more amino acid residues present within 10 Å from a calcium and/or sodium ion identified in the three-dimensional Termamyl-like α-amylase structure in such a manner that the affinity of the α-amylase for calcium is increased.

The amino acid residues found within a distance of 10 Å from the Ca^{2+} binding sites of the *B. licheniformis* α-amylase with the amino acid sequence SEQ ID NO 2 were determined as described in Example 2 and are as follows:

V102, I103, N104, H105, K106, R125, W155, W157, Y158, H159, F160, D161, G162, T163, Y175, K176, F177, G178, K180, A181, W182, D183, W184, E185, V186, S187, N192, Y193, D194, Y195, L196, M197, Y198, A199, D200, I201, D202, Y203, D204, H205, P206, V208, A209, D231, A232, V233, K234, H235, I236, K237, F238, F240, L241, A294, A295, S296, T297, Q298, G299, G300, G301, Y302, D303, M304, R305, K306, L307, W342, F343, L346, Q393, Y394, Y396, H405, H406, D407, I408, V409, R413, E414, G415, D416, S417, V419, A420, N421, S422, G423, L424, I428, T429,

D430, G431, P432, V440, G441, R442, Q443, N444, A445, G446, E447, T448, W449, I462, G475, Y480, V481, Q482, R483.

In order to construct a variant according to this aspect of the invention it is desirable to replace at least one of the above mentioned amino acid residues (or an amino acid residue occupying an equivalent position in another Termamyl-like α-amylase than that defined by SEQ ID NO 2), which is contemplated to be involved in providing a non-optimal calcium binding, with any other amino acid residue which improves the Ca^{2+} binding affinity of the variant enzyme. In practice, the identification and subsequent modification of the amino acid residue is performed by the following method:

i) identifying an amino acid residue within 10 Å from a Ca^{2+} binding site of a Termamyl-like α-amylase structure, which from structural or functional considerations is believed to be responsible for a non-optimal calcium ion interaction,

ii) constructing a variant in which said amino acid residue is replaced with another amino acid residue which from structural or functional considerations is believed to be important for establishing a higher Ca^{2+} binding affinity, and testing the Ca^{2+} dependency of the resulting Termamyl-like α-amylase variant.

In the present context, the term "non-optimal calcium ion interaction" is intended to indicate that the amino acid residue in question is selected on the basis of a presumption that substituting said amino acid residue for another may improve a calcium ion binding interaction of the enzyme. For instance, the amino acid residue in question may be selected on the basis of one or more of the following considerations:

- to obtain an improved interaction between a calcium ion and an amino acid residue located near to the surface of the enzyme (as identified from the structure of the Termamyl-like α-amylase). For instance, if the amino acid residue in question is exposed to a surrounding solvent, it may be advantageous to increase the shielding of said amino acid residue from the solvent so as to provide for a stronger interaction between said amino acid residue and a calcium ion. This can be achieved by replacing said residue (or an amino acid residue in the vicinity of said residue contributing to the shielding) by an amino acid residue which is more bulky or otherwise results in an improved shielding effect.

- to stabilize a calcium binding site, for instance by stabilizing the structure of the Termamyl-like α-amylase (e.g. by stabilizing the contacts between the A, B and C domains or stabilizing one or more of the domains as such). This may, e.g., be achieved

by providing for a better coordination to amino acid side chains, which may, e.g., be obtained by replacing an N residue with a D residue and/or a Q residue with an E residue (e.g. N104D), e.g. within 10 Å, and preferably within 3 or 4Å, of a calcium binding site.

- to protect the calcium binding site or to improve the coordination between the calcium ion and the calcium binding site, e.g. by providing a stronger interaction between the ion and the binding site.

Before actually constructing a Termamyl-like α-amylase variant according to the above principles it may be convenient to evaluate the contemplated amino acid modification by its accommodation into the Termamyl-like α-amylase structure, e.g. into a model structure of the parent Termamyl-like α-amylase.

Preferably, the amino acid residue to be modified is located within 8 Å of a Ca^{2+} binding site residue, such as within 5 Å of such residue. The amino acid residues within 8 Å and 5 Å, respectively, may easily be identified by an analogous method used for identifying amino acid residues within 10 Å (cf. Example 2).

The following mutation is contemplated to be of particular interest with respect to decreasing the Ca^{2+} dependency of a Termamyl-like α-amylase:

N104D (of the *B. licheniformis* α-amylase SEQ ID NO 2, or an equivalent (N to D) mutation of an equivalent position in another Termamyl-like α-amylase.)

In connection with substitutions of relevance for Ca^{2+} dependency, some other substitutions appear to be of importance in stabilizing the enzyme conformation (for instance the Domains A-B and/or Domains A-C interactions contributing to the overall stability of the enzyme) in that they may, e.g., enhance the strength of binding or retention of calcium ion or sodium ion at or within a calcium or sodium binding site, respectively, within the parent Termamyl-like α-amylase.

It is desirable to stabilize the C-domain in order to increase the calcium stability and/or thermostability of the enzyme. In this connection the stabilization may result in a stabilization of the binding of calcium by the enzyme, and an improved contact between the C-domain and the A-domain (of importance for thermostability). The latter may be achieved by introduction of cysteine bridges, salt bridges or increase hydrogen, hydrophobic and/or electrostatic interactions.

For instance, the C-domain of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 may be stabilized by introduction of a cysteine bridge between domain A and domain C, e.g. by introducing of the following mutations: A349C+I479C and/or L346C+I430C.

A salt bridge may be obtained by introduction of the following mutations:

N457D,E

N457D,E+K385R

F350D,E+I430R,K

F350D,E+I411R,K

The calcium site of Domain C may be stabilized by replacing the amino acid residues H408 and/or G303 with any other amino acid residue. Of particular interest is the following mutations:

H408Q,E,N,D and/or G303N,D,Q,E

which are contemplated to provide a better calcium binding or protection from calcium depletion.

Similar mutations may be introduced in equivalent positions of other Termamyl-like α-amylases.

Other substitution mutations (relative to *B. licheniformis* α-amylase, SEQ ID No. 2) which appear to be of importance, *inter alia*, in the context of reducing calcium dependency include the following: R23K, H156Y, A181T, A209V and G310D (or equivalent mutations in equivalent positions in another Termamyl-like α-amylase). Substitutions of R214 and P345 with other amino acids may also be of importance in this connection.

<u>Variants with altered activity at higher/lower pH</u>

It is contemplated that it is possible to change the pH optima of a Termamyl-like α-amylase or the enzymatic activity at a given pH by changing the pKa of the active site residues. This may be achieved, e.g. by changing the electrostatic interaction or hydrophobic interaction between functional groups of amino acid side chains of the amino acid residue to be modified and of its close surroundings. This may, e.g., be accomplished by the following method:

i) in a structure of the Termamyl-like α-amylase in question to identifying an amino acid residue within 15 Å from an active site residue, in particular 10 Å from an active

site residue, which amino acid residue is contemplated to be involved in electrostatic or hydrophobic interactions with an active site residue,

ii) replacing, in the structure, said amino acid residue with an amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue and evaluating the accommodation of the amino acid residue in the structure,

iii) optionally repeating step i) and/or ii) until an amino acid replacement has been identified which is accommodated into the structure,

iv) constructing a Termamyl-like α-amylase variant resulting from steps i), ii) and optionally iii) and testing the pH dependent enzymatic activity of interest of said variant.

In the above method it may be of particular relevance to add a positively charged residue within 5 Å of a glutamate (thereby lowering the pKa of the glutamate from about 4.5 to 4), or to add a negatively charged residue within 5 Å of a glutamate (thereby increasing the pKa to about 5), or to make similar modifications within a distance of about 5 Å of a Histidine.

In a further aspect the invention relates to a variant of a Termamyl-like α-amylase which exhibits a higher activity at a lower pH (e.g. compared to the pH optimum) than the parent α-amylase. In particular, the variant comprises a mutation of an amino acid residue corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2):

E336, Q333, P331, I236, V102, A232, I103, L196

The following mutations are of particular interest:

E336R,K

Q333R,K

P331R,K

V102R,K,A,T,S,G;

I236K,R,N;

I103K,R;

L196K,R;

A232T,S,G;

or any combination of two or more of these variants or any combination of one or more of these variants with any of the other variants disclosed herein.

In a still further aspect the invention relates to a variant of a Termamyl-like α-amylase which has a higher activity at a higher pH than the parent α-amylase. In particular, the variant comprises a mutation of an amino acid residue corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2):

N236, H281, Y273

In particular, the variant comprises a mutation corresponding to at least one of the following mutations of the *B. licheniformis* α-amylase (SEQ ID NO 2):

N326I,Y,F,L,V

H281F,I,L

Y273F,W

or any combination of two or more of these variants or any combination of one or more of these variants with any of the other variants disclosed herein.

A mutation which appears to be importance in relation to the specific activity of variants of the invention is a mutation corresponding to the substitution S187D in *B. licheniformis* α-amylase (SEQ ID NO 2).

Variants with increased thermostability and/or altered temperature optimum

In a further desired aspect the invention relates to a variant of a parent Termamyl-like α-amylase, which variant is the result of one or more amino acid residues having been deleted from, replaced or added to the parent α-amylase so as to obtain an increased thermostability of the variant.

The Termamyl-like α-amylase structure contains a number of unique internal holes, which may contain water, and a number of crevices. In order to increase the thermostability of the α-amylase it may be desirable to reduce the number of holes and crevices (or reduce the size of the holes or crevices), e.g. by introducing one or more hydrophobic contacts, preferably achieved by introducing bulkier residues, in the vicinity or surroundings of the hole. For instance, the amino acid residues to be modified are those which are involved in the formation of the hole.

Accordingly, in a further aspect the present invention relates to a method of increasing the thermostability and/or altering the temperature optimum of a parent Termamyl-like α-amylase, which method comprises

i) identifying an internal hole or a crevice of the parent Termamyl-like α-amylase in the three-dimensional structure of said α-amylase,

ii) replacing, in the structure, one or more amino acid residues in the neighborhood of the hole or crevice identified in i) with another amino acid residue which from structural or functional considerations is believed to increase the hydrophobic interaction and to fill out or reduce the size of the hole or crevice,

iii) constructing a Termamyl-like α-amylase variant resulting from step ii) and testing the thermostability and/or temperature optimum of the variant.

The structure used for identifying the hole or crevice of the parent Termamyl-like α-amylase may be the structure identified in Appendix 1 or a model structure of the parent Termamyl-like α-amylase built thereon.

It will be understood that the hole or crevice is identified by the amino acid residues surrounding the hole/crevice, and that modification of said amino acid residues are of importance for filling or reducing the size of the hole/crevice. The particular amino acid residues referred to below are those which in crystal structure have been found to flank the hole/crevice in question.

In order to fill (completely or partly) a major hole located between domain A and B, mutation to any other amino acid residue of an amino acid residue corresponding to one or more of the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) is contemplated:

L61, Y62, F67, K106, G145, I212, S151, R214, Y150, F143, R146

Of particular interest is a mutation to a more bulky amino acid residue than the amino acid residue of the parent enzyme.

Of particular interest is a variant of a Termamyl-like α-amylase which comprises a mutation corresponding to the following mutations (using the numbering of *B. licheniformis* α-amylase (SEQ ID NO 2):

L61W,V,F;

Y62W;

F67W;

K106R,F,W;

G145F,W

I212F,L,W,Y,R,K;

S151 replaced with any other amino acid residue and in particular with F,W,I or L;
R214W;
Y150R,K;
F143W; and/or
R146W.

In order to fill a hole in the vicinity of the active site mutation to any other amino acid residue of an amino acid residue corresponding to one or more of the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) is contemplated:
L241, I236.

Of interest is a mutation to a more bulky amino acid residue.

Of particular interest is a variant of a Termamyl-like α-amylase which comprises a mutation corresponding to one or more of the following mutations in the *B. licheniformis* α-amylase:
L241I,F,Y,W; and/or
I236L,F,W,Y.

In order to fill a hole in the vicinity of the active site mutation to any other amino acid residue of an amino acid residue corresponding to one or more of the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) is contemplated:
L7, V259, F284

Of interest is a mutation to a more bulky amino acid residue.

Of particular interest is a variant of a Termamyl-like α-amylase which comprises a mutation corresponding to one or more of the following mutations in the *B. licheniformis* α-amylase:
L7F,I,W
V259F,I,L
F284W

In order to fill a hole in the vicinity of the active site mutation to any other amino acid residue of an amino acid residue corresponding to one or more of the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) is contemplated:
F350, F343

Of interest is a mutation to a more bulky amino acid residue.

Of particular interest is a variant of a Termamyl-like α-amylase which comprises a mutation corresponding to one or more of the following mutations in the *B. licheniformis* α-amylase:

F350W

F343W

In order to fill a hole in the vicinity of the active site mutation to any other amino acid residue of an amino acid residue corresponding to one or more of the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) is contemplated:

L427, V481

Of interest is a mutation to a more bulky amino acid residue.

Of particular interest is a variant of a Termamyl-like α-amylase which comprises a mutation corresponding to one or more of the following mutations in the *B. licheniformis* α-amylase:

L427F,L,W

V481,F,I,L,W

Variants with an altered cleavage pattern

In the starch liquefaction process it is desirable to use an α-amylase which is capable of degrading the starch molecules into long branched oligo saccharides (like, e.g. the Fungamyl-like α-amylases) rather than shorter branched oligo saccharides (like conventional Termamyl-like α-amylases). The resulting very small branched oligosaccharides (panose precursors) cannot be hydrolyzed properly by pullulanases, which in the liquefaction process are used after the α-amylases and before the amyloglucosidases. Thus, in the presence of panose precursors the action of glucoamylase ends up with a high degree of the small branched limiting-dextrin, the trisaccharide panose. The presence of panose lowers the saccharification yield significantly and is thus undesirable.

Thus, one aim of the present invention is to change the degradation characteristics of a Termamyl-like α-amylase to that of a Fungamyl-like α-amylases without at the same time reducing the thermostability of the Termamyl-like α-amylase.

Accordingly, in a further aspect the invention relates to a variant of a Termamyl-like α-amylase which has a reduced ability to cleave a substrate close to the branching point.

The variant may suitably be constructed by a method which comprises

i) identifying the substrate binding area of the parent Termamyl-like α-amylase in a model of the three-dimensional structure of said α-amylase, (e.g. within a sphere of 4Å from the substrate binding site (as defined in the section above entitled "Substrate Binding Site"),

ii) replacing, in the model, one or more amino acid residues of the substrate binding area of the cleft identified in i), which is/are believed to be responsible for the cleavage pattern of the parent α-amylase, with another amino acid residue which from structural considerations is believed to result in an altered substrate cleavage pattern, or deleting one or more amino acid residues of the substrate binding area contemplated to introduce favorable interactions to the substrate or adding one or more amino acid residues to the substrate binding area contemplated to introduce favorable interactions to the substrate, and

iii) constructing a Termamyl-like α-amylase variant resulting from step ii) and testing the substrate cleavage pattern of the variant.

Of particular interest is a variant which cleaves an amylopectin substrate, from the reducing end, more than one glucose unit from the branching point, preferably more than two or three glucose units from the branching point, i.e. at a further distance from the branching point than that obtained by use of a wild type *B. licheniformis* α-amylase.

Residues of particular interest in connection with this aspect of the invention correspond to the following residues of the *B. licheniformis* α-amylase (SEQ ID NO 2): V54, D53, Y56, Q333, G57, and the variants according to this aspect preferably comprises a mutation in one or more of these residues.

In particular, the variant comprises at least one of the following mutations, which are expected to prevent cleavage close to the branching point:

V54L,I,F,Y,W,R,K,H,E,Q

D53L,I,F,Y,W

Y56W

Q333W

G57all possible amino acid residues

A52amino acid residues larger than A, e.g. A52W,Y,L,F,I.

Variants of a fungal α-amylase

In a still further embodiment the invention relates to a variant of a parent Fungamyl-like α-amylase, in which variant at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to amino acid residues 291-313 of the amino acid sequence of SEQ ID No. 10, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 98-210 of the amino acid sequence shown in SEQ ID No. 4, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 4 or a corresponding part of another Termamyl-like α-amylase as a template.

For instance, the variant may be one, in which the amino acid fragment X-Y of the parent α-amylase, which corresponds to or is within the amino acid fragment 117-185 of SEQ ID No. 10, has/have been replaced with an amino acid fragment Z-V, which corresponds to or is within the amino acid fragment 98-210 of the amino acid sequence shown in SEQ ID No. 4, in which variant, X is an amino acid residue corresponding to the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 10, Y is an amino acid residue corresponding to the amino acid occupying position 181, 182, 183, 184 or 185 of SEQ ID No. 10, Z is an amino acid residue corresponding to the amino acid occupying position 98, 99, 100, 101 or 102 of SEQ ID No. 4, and V is an amino acid residue corresponding to the amino acid occupying position 206, 207, 208, 209 or 210 of SEQ ID No. 4.

A specific example of a variant according to this aspect of the invention is one, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-181 of SEQ ID No. 10, has been replaced with the amino acid fragment corresponding to amino acid residues 102-206 of the amino acid sequence shown in SEQ ID No. 4.

Another example of a variant according to this aspect of the invention is one, in which the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-174 of SEQ ID No. 10, has been replaced with the amino acid fragment corresponding to amino acid residues 102-199 of the amino acid sequence shown in SEQ ID No. 4.

In a further embodiment the invention relates to a variant of a parent Fungamyl-like α-amylase, in which an amino acid fragment corresponding to amino acid residues 181-184 of the amino acid sequence shown in SEQ ID No. 10 has been deleted.

General mutations in variants of the invention

It may be preferred that the variant of the invention or prepared in accordance with the method of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues present in the part of the α-amylase variant having been modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

Analogously, it may be preferred that one or more cysteine residues present in the amino acid residues with which the parent α-amylase is modified are replaced with a non-cysteine residues such as serine, alanine, threonine, glycine, valine or leucine.

Furthermore, the variant of the invention may either as the only modification or in combination with any of the above outlined modifications be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID No. 8 is replaced by an Asn and/or Gln, respectively. Also of interest is the modification of one or more of the Lys residues present in the Termamyl-like α-amylase is replaced by an Arg present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID No. 8 is replaced by an Asn and/or Gln, respectively.

It will be understood that in accordance with the present invention variants may be prepared which carry two or more of the above outlined modifications. For instance, variants may be prepared which comprises a modification in the loop 1 and loop 2 region, a modification in loop 2 and limited loop 3, a modification in loop 1, loop 2, loop 3 and loop 8, etc.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

Methods of preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard

techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

For region-specific random mutagenesis with a view to improving the thermal stability of a parent Termamyl-like α-amylase, codon positions corresponding to the following amino acid residues of the *B. licheniformis* α-amylase (SEQ ID NO 2) may appropriately be targeted:

To improve the stability of the calcium site between Domain A and C

I428-A435

T297-L308

F403-V409

To improve the stability between domain A and B:

D180-D204

H156-T163

A232-F238

With a view to achieving improved binding of a substrate (i.e. improved binding of a carbohydrate species, such as amylose or amylopectin) by a Termamyl-like α-amylase variant, modified (e.g. higher) substrate specificity and/or modified (e.g. higher) specificity with respect to cleavage (hydrolysis) of substrate, it appears that the following codon positions for the amino acid sequence shown in SEQ ID NO 2 (or equivalent codon positions for another parent Termamyl-like α-amylase in the context of the invention) may particularly appropriately be targeted:

13-18

50-56

70-76

102-109

163-172

189-199

229-235

360-264

327-335

The random mutagenesis of a DNA sequence encoding a parent α-amylase to be performed in accordance with step a) of the above-described method of the invention may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by

subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the amylolytic enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α-amylase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the amylolytic enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent amylolytic enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to

the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harbored in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step (b) or the screening step (c) being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

<u>Localized random mutagenesis</u>: the random mutagenesis may advantageously be localized to a part of the parent α-amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above or any other suitable technique known in the art.

Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently performed by use of aa filter assay based on the following principle:

A microorganism capable of expressing the mutated amylolytic enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The top filter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore^{TM}. The filter may be pretreated with any of the conditions to be used for screening or may be treated during the detection of enzymatic activity.

The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

α-Amylase activity is detected by Cibacron Red labelled amylopectin, which is immobilized on agarose. For screening for variants with increased thermal and high-pH stability, the filter with bound α-amylase variants is incubated in a buffer at pH 10.5 and 60° or 65°C for a specified time, rinsed briefly in deionized water and placed on the amylopectin-agarose matrix for activity detection. Residual activity is seen as lysis of

Cibacron Red by amylopectin degradation. The conditions are chosen to be such that activity due to the α-amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected. Stabilized variants show, under the same conditions, increased color intensity due to increased liberation of Cibacron Red.

For screening for variants with an activity optimum at a lower temperature and/or over a broader temperature range, the filter with bound variants is placed directly on the amylopectin-Cibacron Red substrate plate and incubated at the desired temperature (e.g. 4°C, 10°C or 30°C) for a specified time. After this time activity due to the α-amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected, whereas variants with optimum activity at a lower temperature will show increase amylopectin lysis. Prior to incubation onto the amylopectin matrix, incubation in all kinds of desired media - e.g. solutions containing Ca^{2+}, detergents, EDTA or other relevant additives - can be carried out in order to screen for changed dependency or for reaction of the variants in question with such additives.

Testing of variants of the invention

The testing of variants of the invention may suitably be performed by determining the starch-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying starch-degrading host cells. Further testing as to altered properties (including specific activity, substrate specificity, cleavage pattern, thermoactivation, pH optimum, pH dependency, temperature optimum, and any other parameter) may be performed in accordance with methods known in the art.

Expression of α-amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected

to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus*

selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α-amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli*. The transformation of the

bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

The α-amylase variants of this invention possesses valuable properties allowing for various industrial applications. In particular the enzyme variants finds potential applications as a component in washing, dishwashing and hard surface cleaning detergent compositions, but it may also be useful in the production of sweeteners and ethanol from starch and for textile desizing. Conditions for conventional starch converting processes and

liquefaction and/or saccharification processes are described in for instance US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

Production of sweeteners from starch: A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes, viz. a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to dextrins by an α-amylase (e.g. Termamyl™) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2 h. In order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrins are converted into dextrose by addition of a glucoamylase (e.g. AMG™) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g. Promozyme™). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C), and the liquefying α-amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucose isomerase (such as Sweetzyme™).

At least 3 enzymatic improvements of this process could be obtained. All three improvements could be seen as individual benefits, but any combination (e.g. 1+2, 1+3, 2+3 or 1+2+3) could be employed:

Improvement 1. Reduction of the calcium dependency of the liquefying alpha-amylase.

Addition of free calcium is required to ensure adequately high stability of the α-amylase, but free calcium strongly inhibits the activity of the glucose isomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium to below 3-5 ppm. Cost savings could be obtained if such an operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α-amylase which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α-amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Improvement 2. Reduction of formation of unwanted Maillard products

The extent of formation of unwanted Maillard products during the liquefaction process is dependent on the pH. Low pH favors reduced formation of Maillard products. It would thus be desirable to be able to lower the process pH from around pH 6.0 to a value around pH 4.5; unfortunately, all commonly known, thermostable Termamyl-like α-amylases are not very stable at low pH (i.e. pH < 6.0) and their specific activity is generally low.

Achievement of the above-mentioned goal requires a Termamyl-like α-amylase which is stable at low pH in the range of 4.5-5.5 and at free calcium concentrations in the range of 0-40 ppm, and which maintains a high specific activity.

Improvement 3.

It has been reported previously (US patent 5,234,823) that when saccharifying with *A. niger* glucoamylase and *B. acidopullulyticus* pullulanase, the presence of residual α-amylase activity from the liquefaction process can lead to lower yields of dextrose if the α-amylase is not inactivated before the saccharification stage. This inactivation can typically be carried out by adjusting the pH to below 4.3 at 95°C, before lowering the temperature to 60°C for saccharification.

The reason for this negative effect on dextrose yield is not fully understood, but it is assumed that the liquefying α-amylase (for example Termamyl™ 120 L from *B. licheniformis*) generates "limit dextrins" (which are poor substrates for *B. acidopullulyticus* pullulanase) by hydrolyzing 1,4-alpha-glucosidic linkages close to and on both sides of the branching points in amylopectin. Hydrolysis of these limit dextrins by glucoamylase leads to a build-up of the trisaccharide panose, which is only slowly hydrolyses by glucoamylase.

The development of a thermostable α-amylase which does not suffer from this disadvantage would be a significant process improvement, as no separate inactivation step would be required.

If a Termamyl-like, low-pH-stable α-amylase is developed, an alteration of the specificity could be an advantage needed in combination with increased stability at low pH.

The methodology and principles of the present invention make it possible to design and produce variants according to the invention having the required properties as outlined above.

Detergent Compositions

According to the invention, the α-amylase may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g. as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000, ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% of water and 0-30% of organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate,

alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as lipase, cutinase, protease, cellulase, peroxidase, e.g., laccase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxy acids of e.g. the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative as e.g. an aromatic borate ester, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18}))	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaA1SiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18}))	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaA1SiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaA1SiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaA1SiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaA1SiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluene sulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaA1SiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The α-amylase variant of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the α-amylase may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of α-amylase per liter of wash liquor.

Dishwashing Composition

The dishwashing detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will contain 0-90% of non-ionic surfactant such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains 1-90% of detergent builders.

Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts especially alkali metal pyrophosphates, orthophosphates, and polyphosphates. An example of phosphorus-containing organic alkaline detergent builder, when present, includes the water-soluble salts of phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates and silicates as well as the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best-known representatives.

Examples of suitable organic builders include the alkali metal, ammonium and substituted ammonium, citrates, succinates, malonates, fatty acid sulphonates, carboxymetoxy succinates, ammonium polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates, polyacetyl carboxylates and polyhydroxsulphonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated

trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g. propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

The dishwashing detergent composition of the invention may also contain other conventional detergent ingredients, e.g. deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners and perfumes.

Finally, the α-amylase variant of the invention may be used in conventional dishwashing detergents, e.g. in any of the detergents described in any of the following patent publications:

EP 518719, EP 518720, EP 518721, EP 516553, EP 516554, EP 516555, GB 2200132, DE 3741617, DE 3727911, DE 4212166, DE 4137470, DE 3833047, WO 93/17089, DE 4205071, WO 52/09680, WO 93/18129, WO 93/04153, WO 92/06157, WO 92/08777, EP 429124, WO 93/21299, US 5141664, EP 561452, EP 561446, GB 2234980, WO 93/03129, EP 481547, EP 530870, EP 533239, EP 554943, EP 346137, US 5112518, EP 318204, EP 318279, EP 271155, EP 271156, EP 346136, GB 2228945, CA 2006687, WO 93/25651, EP 530635, EP 414197, US 5240632.

EXAMPLES

EXAMPLE 1

Example on Homology building of TERM

The overall homology of the *B. licheniformis* α-amylase (in the following referred to as TERM) to other Termamyl-like α-amylases is high and the percent similarity is extremely high. The similarity calculated for TERM to BSG (the *B. stearothermophilus* α-amylase with SEQ ID NO 6), and BAN (the *B. amyloliquefaciens* α-amylase with SEQ ID NO 4) using the University of Wisconsin Genetics Computer Group's program GCG gave 89% and 78%, respectively. TERM has a deletion of 2 residues between residue G180 and K181 compared to BAN and BSG. BSG has a deletion of 3 residues between G371 and I372 in comparison with BAN and TERM. Further BSG has a C-terminal extension of more than 20 residues compared to BAN and TERM. BAN has 2 residues less and TERM has one residue less in the N-terminal compared to BSG.

The structure of the *B. licheniformis* (TERM) and of the *B. amyloliquefaciens* α-amylase (BAN), respectively, was model built on the structure disclosed in Appendix 1 herein. The structure of other Termamyl-like α-amylases (e.g. those disclosed herein) may be built analogously.

In comparison with the α-amylase used for elucidating the present structure, TERM differs in that it lacks two residues around 178-182. In order to compensate for this in the model structure, the HOMOLOGY program from BIOSYM was used to substitute the residues in equivalent positions in the structure (not only structurally conserved regions) except for the deletion point. A peptide bond was established between G179(G177) and K180(K180) in TERM(BAN). The close structural relationship between the solved structure and the model structure (and thus the validity of the latter) is indicated by the presence of only very few atoms found to be too close together in the model.

To this very rough structure of TERM was then added all waters (605) and ions (4 Calcium and 1 Sodium) from the solved structure (Appendix 1) at the same coordinates as for said solved structure using the INSIGHT program. This could be done with only few overlaps - in other words with a very nice fit. This model structure were then minimized using 200 steps of Steepest descent and 600 steps of Conjugated gradient (see Brooks et al 1983, J. Computational Chemistry 4, p.187-217). The minimized structure was then subjected to molecular dynamics, 5ps heating followed by up to 200ps equilibration but

more than 35ps. The dynamics as run with the Verlet algorithm and the equilibration temperature 300K were kept using the Behrendsen coupling to a water bath (Berendsen et. al., 1984, J. Chemical Physics 81, p. 3684-3690). Rotations and translations were removed every picosecond. The potential energy became stable after appr. 35ps equilibration. A mean dynamics structure was extracted and can be used for further analysis.

EXAMPLE 2

Determination of residues within 10 Å from the ions present in the solved structure

The coordinates of Appendix 1 are read into the INSIGHT program provided by BIOSYM technologies. The spatial coordinates are presented showing the bonds between the atoms. The ions are presented as well as the water atoms. The program package part of creating subset are used to create a 10 Å subset around the Calcium and the Sodium ions in the structure using the command ZONE. All residues having an atom within the 10 Å are compiled and written out by the LIST MOLECULE command. By giving the ions the name ium in the coordinate file a 10 Å sphere around all atoms called ium is compiled. The specific residues identified in this manner are given further above in the section entitled "Ca^{2+} dependency".

EXAMPLE 3

Determination of cavities in the solved structure (Appendix 1)

The solved structure exhibits many internal holes and cavities. When analyzing for such cavities the Connolly program is normally used (Lee, B. and Richards, F.M. (1971) J. Mol. Biol. 55, p. 379-400). The program uses a probe with radius to search the external and internal surface of the protein. The smallest hole observable in this way has the probe radius.

To analyze the solved structure a modified version of the Connolly program included in the program of INSIGHT were used. First the water molecules and the ions were removed by unmerging these atoms from the solved structure. By using the command MOLECULE SURFACE SOLVENT the solvent accessible surface area were calculated for all atoms and residues using a probe radius of 1.4 Å, and displayed on the graphics screen together with the model of the solved structure. The internal cavities where then seen as dot surfaces with no connections to external surface.

Mutant suggestions for filling out the holes are given in the specification (in the section entitled "Variants with increased thermostability and/or altered temperature optimum"). By using the homology build structures or/and the sequence alignment mutations for the homologous structures of TERM and BSG and BAN can be made.

EXAMPLE 4

Construction of Termamyl™ variants in accordance with the invention

Termamyl (SEQ ID NO. 2) is expressed in *B. subtilis* from a plasmid denoted pDN1528. This plasmid contains the complete gene encoding Termamyl, *amyL*, the expression of which is directed by its own promoter. Further, the plasmid contains the origin of replication, *ori*, from plasmid pUB110 and the *cat* gene from plasmid pC194 conferring resistance towards chloramphenicol. pDN1528 is shown in Fig. 9.

A specific mutagenesis vector containing a major part of the coding region of SEQ ID NO 1 was prepared. The important features of this vector, denoted pJeEN1, include an origin of replication derived from the pUC plasmids, the *cat* gene conferring resistance towards chloramphenicol, and a frameshift-containing version of the *bla* gene, the wild type of which normally confers resistance towards ampicillin (amp^R phenotype). This mutated version results in an amp^S phenotype. The plasmid pJeEN1 is shown in Fig. 10, and the *E. coli* origin of replication, *ori*, *bla*, *cat* , the 5'-truncated version of the Termamyl amylase gene, and selected restriction sites are indicated on the plasmid.

Mutations are introduced in *amyL* by the method described by Deng and Nickoloff (1992, Anal. Biochem. 200, pp. 81-88) except that plasmids with the "selection primer" (primer #6616; see below) incorporated are selected based on the amp^R phenotype of transformed *E. coli* cells harboring a plasmid with a repaired *bla* gene, instead of employing the selection by restriction enzyme digestion outlined by Deng and Nickoloff. Chemicals and enzymes used for the mutagenesis were obtained from the Chameleon™ mutagenesis kit from Stratagene (catalogue number 200509).

After verification of the DNA sequence in variant plasmids, the truncated gene, containing the desired alteration, is subcloned into pDN1528 as a *Pst*I-*Eco*RI fragment and transformed into a protease- and amylase-depleted *Bacillus subtilis* strain in order to express the variant enzyme.

The Termamyl variant V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CGC TTG

The Termamyl variant A52W + V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CCA TTG GCT CG

Primer #6616 (written 5' to 3', left to right; P denotes a 5' phosphate):

P CTG TGA CTG GTG AGT ACT CAA CCA AGT C

EXAMPLE 5

Saccharification in the presence of "residual" α-amylase activity

Two appropriate Termamyl variants with altered specificity were evaluated by saccharifying a DE 10 (DE = dextrose equivalent) maltodextrin substrate with *A. niger* glucoamylase and *B. acidopullulyticus* pullulanase under conditions where the variant amylase was active.

Saccharification: Substrates for saccharification were prepared by dissolving 230 g DE 10 spray-dried maltodextrin, prepared from common corn starch, in 460 ml boiling deionized water and adjusting the dry substance (DS) content to approximately 30% w/w. The pH was adjusted to 4.7 (measured at 60°C) and aliquots of substrate corresponding to 15 g dry weight were transferred to 50 ml blue cap glass flasks.

The flasks were then placed in a shaking water bath equilibrated at 60°C, and the enzymes added. The pH was readjusted to 4.7 where necessary.

The following enzymes were used:

Glucoamylase: AMG™ (Novo Nordisk A/S); dosage 0.18 AG/g DS
Pullulanase: Promozyme™ (Novo Nordisk A/S); dosage 0.06 PUN/g DS
α-Amylases: Termamyl™ (Novo Nordisk A/S); dosage 60 NU/g DS
Termamyl variant V54W; dosage 60 NU/g DS
Termamyl variant V54W + A52W; dosage 60 NU/g DS

2 ml samples were taken periodically. The pH of each sample was adjusted to about 3.0, and the sample was then heated in a boiling water bath for 15 minutes to inactivate the enzymes. After cooling, the samples were treated with approximately 0.1 g

mixed-bed ion exchange resin (BIO-Rad 501-X (D)) for 30 minutes on a rotary mixer and then filtered. The carbohydrate composition of each sample was determined by HPLC. The following results were obtained after 72 hours [DP_n denotes a dextrose (D-glucose) oligomer with n glucose units]:

α-amylase	$\%DP_1$	$\%DP_2$	$\%DP_3$	$\%DP_4$
None (control)	95.9	2.8	0.4	1.0
V54W	96.0	2.9	0.4	0.8
V54W + A52W	95.9	2.8	0.4	0.8
Termamyl™	95.6	2.8	0.8	0.8

It can be seen from the above results that compared with the control (no α-amylase activity present during liquefaction), the presence of α-amylase activity from variants V54W and V54W + A52W did not lead to elevated panose (DP_3) levels. In contrast, Termamyl α-amylase activity resulted in higher levels of panose and a subsequent loss of D-glucose (DP_1) yield.

Thus, if α-amylase variants V54W or V54W + A52W are used for starch liquefaction, it will not be necessary to inactivate the residual α-amylase activity before the commencement of saccharification.

EXAMPLE 6

Calcium-binding affinity of α-amylase variants of the invention

Unfolding of amylases by exposure to heat or to denaturants such as guanidine hydrochloride is accompanied by a decrease in fluorescence. Loss of calcium ions leads to unfolding, and the affinity of α-amylases for calcium can be measured by fluorescence measurements before and after incubation of each α-amylase (e.g. at a concentration of 10 μg/ml) in a buffer (e.g. 50 mM HEPES, pH 7) with different concentrations of calcium (e.g. in the range of 1 μM-100 mM) or of EGTA (e.g. in the range of 1-1000 μM) [EGTA = 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid] for a sufficiently long period of time (such as 22 hours at 55°C).

The measured fluorescence F is composed of contributions form the folded and unfolded forms of the enzyme. The following equation can be derived to describe the dependence of F on calcium concentration ([Ca]):

$$F = [Ca]/(K_{diss} + [Ca])(\alpha_N - \beta_N\log([Ca])) + K_{diss}/(K_{diss} + [Ca])(\alpha_U - \beta_U\log([Ca]))$$

where α_N is the fluorescence of the native (folded) form of the enzyme, β_N is the linear dependence of α_N on the logarithm of the calcium concentration (as observed experimentally), α_U is the fluorescence of the unfolded form and β_U is the linear dependence of α_U on the logarithm of the calcium concentration. K_{diss} is the apparent calcium-binding constant for an equilibrium process as follows:

$$\text{N-Ca} \overset{K_{diss}}{\leftrightarrow} \text{U} + \text{Ca} \qquad (\text{N} = \text{native enzyme; U} = \text{unfolded enzyme})$$

In fact, unfolding proceeds extremely slowly and is irreversible. The rate of unfolding is a dependent on calcium concentration, and the dependency for a given α-amylase provides a measure of the Ca-binding affinity of the enzyme. By defining a standard set of reaction conditions (e.g. 22 hours at 55°C), a meaningful comparison of K_{diss} for different α-amylases can be made. The calcium dissociation curves for α-amylases in general can be fitted to the equation above, allowing determination of the corresponding values of K_{diss}.

The following values for K_{diss} were obtained for a parent Termamyl-like α-amylase having the amino acid sequence shown in SEQ ID No. 1 of WO 95/26397 and for the indicated variant thereof according to the invention:

α-Amylase	K_{diss} (mol/l)
L351C + M430C + T183* + G184*	1.7 ($\pm$0.5) x 10^{-3}
Parent	3.5 ($\pm$1.1) x 10^{-1}

It is apparent from the above that the calcium-binding affinity of the variant in question binds calcium significantly more strongly than the parent, and thereby has a correspondingly lower calcium dependency than the parent.

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SEQUENCE LISTING

In the following SEQ ID Nos. 1, 3, 5 the 5', coding sequence and 3' sequence of the relevant α-amylase genes are illustrated. The 5' sequence is the first separate part of the sequence written with lower case letters, the coding sequence is the intermediate part of the sequence, where the signal sequence is written with lower case letters and the sequence encoding the mature α-amylase is written with upper case letters, and the 3' sequence is the third separate part of the sequence written with lower case letters.

SEQ ID No. 1

cggaagattggaagtacaaaaataagcaaaagattgtcaatcatgtcatgagccatgcgg-gagacggaaaaatcgtctta
atgcacgatatttatgcaacgttcgcagatgctgctgaa-gagattattaaaaagctgaaagcaaaaggctatcaattggt
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CATTGGAGGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
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CCCGCGACATTAACGTTTACGGGGAT
GTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
GAAGTCGATCCCGCTGACCGCAACCG
CGTAATTTCAGGAGAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGG
GCGCGGCAGCACATACAGCGATTTTA
AATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGA
ACCGCATCTATAAGTTTCAAGGAAAG
GCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
GCCGACATCGATTTATGACCATCCTGA

TGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATT
G G A C G G T T T C C G T C T T G A T G C T G T C A
AACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAA
C G G G G A A G G A A A T G T T T A C G G T A G C T
GAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
T T T A A T C A T T C A G T G T T T G A C G T G C C
GCTTCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAG
G A A A T T G C T G A A C G G T A C G G T C G T T T
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G G C A A T C G C T T G A G T C G A C T G T C C A A
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C T C A G G T T T T C T A C G G G G A T A T G T A
CGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTG
A A C C G A T C T T A A A A G C G A G A A A A C A G T
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C A A G G G A A G G C G A C A G C T C G G T T G C A
AATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAAT
G T A T G T C G G C C G G C A A A A C G C C G G T G A
GACATGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGA
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aagagcagagaggacggatttcctgaaggaaatccgtttttttatttt

SEQ ID No. 2
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DGTDWDESRKLNRIYKFQGKAWDWEVSNENGNYDYLMYAD
IDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF
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GRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIY
VQR

SEQ ID No. 3

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atgattcaaaaacgaaagcggacagtttcgttcagacttgtgcttatgtgcacgctgttatttgtcagttt
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C G A A C G A C G G C C A G C A T T
GGAAACGATTGCAGAATGATGCGGAACATTTATCGGATATCGGAATCACTGCCG
T C T G G A T T C C T C C C G C A T A C A A A G G A
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T T C C A G C A A A A A G G G A C G G T C A G A A C
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G G A A A C A C G T A C A G T G A T T T T A A A T G
GCATTGGTATCATTTCGACGGAGCGGACTGGGATGAATCCCGGAAGATCAGCCG
C A T C T T T A A G T T T C G T G G G G A A G G A A
AAGCGTGGGATTGGGAAGTATCAAGTGAAAACGGCAACTATGACTATTTAATGT
A T G C T G A T G T T G A C T A C G A C C A C C C T
GATGTCGTGGCAGAGACAAAAAAATGGGGTATCTGGTATGCGAATGAACTGTCA
T T A G A C G G C T T C C G T A T T G A T G C C G C
CAAACATATTAAATTTTCATTTCTGCGTGATTGGGTTCAGGCGGTCAGACAGGC
G A C G G G A A A A G A A A T G T T T A C G G T T G
CGGAGTATTGGCAGAATAATGCCGGGAAACTCGAAAACTACTTGAATAAAACA

A G C T T T A A T C A A T C C G T G T T T G A T G T T
CCGCTTCATTTCAATTTACAGGCGGCTTCCTCACAAGGAGGCGGATATGATATG
A G G C G T T T G C T G G A C G G T A C C G T T G T
GTCCAGGCATCCGGAAAAGGCGGTTACATTTGTTGAAAATCATGACACACAGCC
G G G A C A G T C A T T G G A A T C G A C A G T C C
AAACTTGGTTTAAACCGCTTGCATACGCCTTTATTTTGACAAGAGAATCCGGTTA
T C C T C A G G T G T T C T A T G G G G A T A T G
TACGGGACAAAAGGGACATCGCCAAAGGAAATTCCCTCACTGAAAGATAATAT
A G A G C C G A T T T T A A A A G C G C G T A A G G A
GTACGCATACGGGCCCCAGCACGATTATATTGACCACCCGGATGTGATCGGATG
G A C G A G G G A A G G T G A C A G C T C C G C C G
CCAAATCAGGTTTGGCCGCTTTAATCACGGACGGACCCGGCGGATCAAAGCGGA
T G T A T G C C G G C C T G A A A A A T G C C G G C
GAGACATGGTATGACATAACGGGCAACCGTTCAGATACTGTAAAAATCGGATCT
G A C G G C T G G G G A G A G T T T C A T G T A A A
CGATGGGTCCGTCTCCATTTATGTTCAGAAATAA
g g t a a t a a a a a a a c a c c t c c a a g c t g a g t g c g g g t a t c a g c t t g g a
ggtgcgtttatttttcagccgtatgacaaggtcggcatcaggtgtgacaaatacggtatgctggctgtcataggtgaca
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SEQ ID No. 4
VNGTLMQYFEWYTPNDGQHWKRLQNDAEHLSDIGITAVWI
PPAYKGLSQSDNGYGPYDLYDLGEFQQKGTVRTKYGTKSE
LQDAIGSLHSRNVQVYGDVVLNHKAGADATEDVTAVEVNP
ANRNQETSEEYQIKAWTDFRFPGRGNTYSDFKWHWYHFDG
ADWDESRKISRIFKFRGEGKAWDWEVSSENGNYDYLMYAD
VDYDHPDVVAETKKWGIWYANELSLDGFRIDAAKHIKFSF
LRDWVQAVRQATGKEMFTVAEYWQNNAGKLENYLNKTSFN
QSVFDVPLHFNLQAASSQGGGYDMRRLLDGTVVSRHPEKA
VTFVENHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQ
VFYGDMYGTKGTSPKEIPSLKDNIEPILKARKEYAYGPQH

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GLKNAGETWYDITGNRSDTVKIGSDGWGEFHVNDGSVSIY

SEQ ID No. 5

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gacagcccgccaaggctGCCGCACCGTTTAACGGCACCATGATGCAGTATTTTGAATGGT
A C T T G C C G G A T G A T G G C A C G
TTATGGACCAAAGTGGCCAATGAAGCCAACAACTTATCCAGCCTTGGCATCACC
G C T C T T T G G C T G C C G C C C G C T T A C A A
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G C G A A T T C A A T C A A A A A G G G A C C G T C C
GCACAAAATACGGAACAAAAGCTCAATATCTTCAAGCCATTCAAGCCGCCCACG
C C G C T G G A A T G C A A G T G T A C G C C G A T
GTCGTGTTCGACCATAAAGGCGGCGCTGACGGCACGGAATGGGTGGACGCCGTC
G A A G T C A A T C C G T C C G A C C G C A A C C A
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G C G G G G C A A C A C C T A C T C C A G C T T T A
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G C C G C A T T T A C A A A T T C C G C G G C A T C
GGCAAAGCGTGGGATTGGGAAGTAGACACGGAAAACGGAAACTATGACTACTT
A A T G T A T G C C G A C C T T G A T A T G G A T C A
TCCCGAAGTCGTGACCGAGCTGAAAAACTGGGGGAAATGGTATGTCAACACAA
C G A A C A T T G A T G G G T T C C G G C T T G A T G
CCGTCAAGCATATTAAGTTCAGTTTTTTTCCTGATTGGTTGTCGTATGTGCGTTC
T C A G A C T G G C A A G C C G C T A T T T A C C
GTCGGGGAATATTGGAGCTATGACATCAACAAGTTGCACAATTACATTACGAAA
A C A G A C G G A A C G A T G T C T T T G T T T G A
TGCCCCGTTACACAACAAATTTTATACCGCTTCCAAATCAGGGGGCGCATTTGA
T A T G C G C A C G T T A A T G A C C A A T A C T C
TCATGAAAGATCAACCGACATTGGCCGTCACCTTCGTTGATAATCATGACACCG

A A C C C G G C C A A G C G C T G C A G T C A T G G
GTCGACCCATGGTTCAAACCGTTGGCTTACGCCTTTATTCTAACTCGGCAGGAA
G G A T A C C C G T G C G T C T T T T A T G G T G A
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G C T C C T C A T C G C G C G C A G G G A T T A T G
CTTACGGAACGCAACATGATTATCTTGATCACTCCGACATCATCGGGTGGACAA
G G G A A G G G G G C A C T G A A A A A C C A G G A
TCCGGACTGGCCGCACTGATCACCGATGGGCCGGGAGGAAGCAAATGGATGTA
C G T T G G C A A A C A A C A C G C T G G A A A A G T
GTTCTATGACCTTACCGGCAACCGGAGTGACACCGTCACCATCAACAGTGATGG
A T G G G G G G A A T T C A A A G T C A A T G G C G
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G A T C A C A A C C C G A C C G T G G A C T G G T
GAATTCGTCCGTTGGACCGAACCACGGTTGGTGGCATGGCCTTGA
tgcctgcga

SEQ ID No. 6
AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITA
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VNPSDRNQEISGTYQIQAWTKFDFPGRGNTYSSFKWRWYH
FDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
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FSFFPDWLSYVRSQTGKPLFTVGEYWSYDINKLHNYITKT
DGTMSLFDAPLHNKFYTASKSGGAFDMRTLMTNTLMKDQP
TLAVTFVDNHDTEPGQALQSWVDPWFKPLAYAFILTRQEG
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SEQ ID No. 10

1 ATPADWRSQS IYFLLTDRFA RTDGSTTATC
31 NTADQKYCGG TWQGIIDKLD YIQGMGFTAI
61 WITPVTAQLP QTTAYGDAYH GYWQQDIYSL
91 NENYGTADDL KALSSALHER GMYLMVDVVA
121 NHMGYDGAGS SVDYSVFKPF SSQDYFHPFC
151 FIQNYEDQTQ VEDCWLGDNT VSLPDLDTTK
181 DVVKNEWYDW VGSLVSNYSI DGLRIDTVKH
211 VQKDFWPGYN KAAGVYCIGE VLDGDPAYTC
241 PYQNVMDGVL NYPIYYPLLN AFKSTSGSMD
271 DLYNMINTVK SDCPDSTLLG TFVENHDNPR
301 FASYTNDIAL AKNVAAFIIL NDGIPIIYAG
331 QEQHYAGGND PANREATWLS GYPTDSELYK
361 LIASANAIRN YAISKDTGFV TYKNWPIYKD
391 DITIAMRKGT DGSQIVTILS NKGASGDSYT
421 LSLSGAGYTA GQQLTEVIGC TTVTVGSDGN
451 VPVPMAGGLP RVLYPTEKLA GSKICSSS

CLAIMS

1. A method of constructing a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity and at least one altered property as compared to said parent α-amylase, which method comprises

i) analyzing the structure of the parent Termamyl-like α-amylase to identify at least one amino acid residue or at least one structural part of the Termamyl-like α-amylase structure, which amino acid residue or structural part is believed to be of relevance for altering said property of the parent Termamyl-like α-amylase (as evaluated on the basis of structural or functional considerations),

ii) constructing a Termamyl-like α-amylase variant, which as compared to the parent Termamyl-like α-amylase, has been modified in the amino acid residue or structural part identified in i) so as to alter said property, and

iii) testing the resulting Termamyl-like α-amylase variant for said property.

2. The method according to claim 1, wherein the property to be altered is selected from the group consisting of substrate specificity, substrate binding, substrate cleavage pattern, temperature stability, pH dependent activity, pH dependent stability (especially increased stability at low (e.g. $pH<6$) or high (e.g. $pH>9$) pH values), stability towards oxidation, Ca^{2+}-dependency and specific activity.

3. The method according to claim 1, wherein the property to be altered is the calcium ion dependency and the structural part to be modified is selected from the group consisting of the C domain, the interface between the A and B domain, the interface between the A and C domain, or the interaction to a calcium binding site of the Termamyl-like α-amylase.

4. The method according to claim 1, wherein the property to be altered is the substrate cleavage pattern and the structural part to be modified is located within 10 Å from an amino acid residue of the substrate binding site.

5. A method of constructing a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity and one or more altered properties as compared to said parent α-amylase, which method comprises

i) comparing the three-dimensional structure of the Termamyl-like α-amylase with the structure of a non-Termamyl-like α-amylase,

ii) identifying a part of the Termamyl-like α-amylase structure which is different from the non-Termamyl-like α-amylase structure and which from structural or functional considerations is contemplated to be responsible for differences in one or more properties of the Termamyl-like and non-Termamyl-like α-amylase, and

iii) modifying the part of the Termamyl-like α-amylase identified in ii) whereby a Termamyl-like α-amylase variant is obtained, one or more properties of which differ from the parent Termamyl-like α-amylase.

6. The method according to claim 5, wherein, in step iii), the part of the Termamyl-like α-amylase is modified so as to resemble the corresponding part of the non-Termamyl-like α-amylase.

7. The method according to claim 5, wherein, in step iii), the modification is accomplished by deleting one or more amino acid residues of the part of the Termamyl-like α-amylase to be modified; by replacing one or more amino acid residues of the part of the Termamyl-like α-amylase to be modified with the amino acid residues occupying corresponding positions in the non-Termamyl-like α-amylase; or by insertion of one or more amino acid residues present in the non-Termamyl-like α-amylase into a corresponding position in the Termamyl-like α-amylase.

8. The method according to claim 5, wherein the non-Termamyl-like α-amylase structure is the structure of a fungal α-amylase or a mammalian α-amylase.

9. The method according to claim 8, wherein the non-Termamyl-like α-amylase is the *Aspergillus oryzae* TAKA α-amylase, the *A. niger* acid α-amylase, the *Bacillus subtilis* α-amylase or the pig pancreatic α-amylase.

10. The method according to claim 1, wherein the parent Termamyl-like α-amylase is derived from a strain of *Bacillus*.

11. The method according to claim 10, wherein the parent α-amylase is derived from a strain of a *B. licheniformis*, *B. amyloliquefaciens*, *B. stearothermophilus* or a strain from an alkalophilic *Bacillus* sp. such as NCIB 12289, NCIB 12512 or NCIB 12513.

12. The method according to claim 1, wherein the parent α-amylase is a hybrid α-amylase comprising a combination of partial amino acid sequences derived from at least two α-amylases, of which one is a Termamyl-like α-amylase and the other(s) are, e.g., from a microbial and/or a mammalian α-amylase.

13. The method according to claim 5, wherein the part of the parent Termamyl-like α-amylase to be modified and identified in step ii) is loop 1, loop 2, loop 3 and/or loop 8 of the parent α-amylase.

14. A method of constructing a variant of a parent Termamyl-like α-amylase, which has a decreased calcium ion dependency as compared to said parent, which method comprises:

i) identifying an amino acid residue within 10 Å from a Ca^{2+} binding site of a Termamyl-like α-amylase in a model of the three-dimensional structure of said α-amylase, which from structural or functional considerations is believed to be responsible for a non-optimal calcium ion interaction,

ii) constructing a variant wherein said amino acid residue is replaced with another amino acid residue which from structural or functional considerations is believed to be important for establishing a higher Ca^{2+} binding affinity, and

iii) testing the Ca^{2+} dependency of the resulting Termamyl-like α-amylase variant.

15. A method of constructing a variant of a parent Termamyl-like α-amylase which variant has α-amylase activity and an altered pH dependent activity, which method comprises

i) in a three-dimensional structure of the Termamyl-like α-amylase in question, identifying an amino acid residue within 15 Å from an active site residue, in particular 10

Å from an active site residue, which amino acid residue is contemplated to be involved in electrostatic or hydrophobic interactions with an active site residue,

ii) replacing, in the structure, said amino acid residue with an amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue and evaluating the accommodation of the amino acid residue in the structure,

iii) optionally repeating step i) and/or ii) until an amino acid replacement has been identified which is accommodated into the structure,

iv) constructing a Termamyl-like α-amylase variant resulting from steps i), ii) and optionally iii) and testing the pH dependent activity of said variant.

16. A method of increasing the thermostability and/or altering the temperature optimum of a parent Termamyl-like α-amylase, which method comprises

i) identifying an internal hole or a crevice of the parent Termamyl-like α-amylase in the three-dimensional structure of said α-amylase,

ii) replacing, in the structure, one or more amino acid residues in the neighborhood of the hole or crevice identified in i) with another amino acid residue which from structural or functional considerations is believed to increase the hydrophobic interaction and to fill out or reduce the size of the hole or crevice,

iii) constructing a Termamyl-like α-amylase variant resulting from step ii) and testing the thermostability and/or temperature optimum of the variant.

17. A method of constructing a variant of a Termamyl-like α-amylase which has a reduced ability to cleave a substrate close to the branching point, which method comprises

i) identifying the substrate binding area of the parent Termamyl-like α-amylase in a model of the three-dimensional structure of said α-amylase,

ii) replacing, in the model, one or more amino acid residues of the substrate binding area of the cleft identified in i), which is/are believed to be responsible for the cleavage pattern of the parent α-amylase, with another amino acid residue which from structural considerations is believed to result in an altered substrate cleavage pattern, or deleting one or more amino acid residues of the substrate binding area contemplated to introduce favorable interactions to the substrate or adding one or more amino acid residues

to the substrate binding area contemplated to introduce favorable interactions to the substrate, and

iii) constructing a Termamyl-like α-amylase variant resulting from step ii) and testing the substrate cleavage pattern of the variant.

18. The method according to claim 1, wherein the α-amylase variant is obtained by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth.

19. A variant of a parent Termamyl-like α-amylase, in which variant, at least one amino acid residue of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 44-57 of the amino acid sequence of SEQ ID No. 4, has been deleted or replaced with one or more amino acid residues which is/are present in a fragment corresponding to the amino acid fragment 66-84 of the amino acid sequence shown in SEQ ID No. 10, or in which variant, one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

20. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid residue occupying position 44, 45, 46, 47 or 48 of SEQ ID No. 4,

Y is the amino acid residue occupying position 51, 52, 53, 54, 55, 56 or 57 of SEQ ID No. 4,

Z is the amino acid residue occupying position 66, 67, 68, 69 or 70 of SEQ ID No. 10, and

V is the amino acid residue occupying position 78, 79, 80, 81, 82, 83 or 84 of SEQ ID No. 10.

21. The variant according to claim 19, wherein X is the amino acid residue occupying position 48 and Y the amino acid residue occupying position 51 of SEQ ID NO 4 and Z is the amino acid residue occupying position 70 and V the amino acid residue occupying position 78 in SEQ ID No 10.

22. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to the amino acid fragment 195-202 of the amino acid sequence of SEQ ID No. 4, has been deleted or replaced with one or more of the amino acid residues which is/are present in an amino acid fragment corresponding to the amino acid fragment 165-177 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

23. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 195 or 196 of SEQ ID No. 4,

Y is the amino acid residue occupying position 198, 199, 200, 201, or 202 of SEQ ID No. 4,

Z is the amino acid residue occupying position 165 or 166 of SEQ ID No. 10, and

V is the amino acid residue occupying position 173, 174, 175, 176 or 177 of SEQ ID No. 10.

24. The variant according to claim 22, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 196-198 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 166-173 of the amino acid sequence shown in SEQ ID No. 10.

25. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 117-185 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to the amino acid fragment 98-210 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

26. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 4,

Y is the amino acid occupying position 181, 182, 183, 184 or 185 of SEQ ID No. 4,

Z is the amino acid occupying position 98, 99, 100, 101, 102 of SEQ ID No. 10, and

V is the amino acid occupying position 206, 207, 208, 209 or 210 of SEQ ID No. 10.

27. The variant according to claim 25, wherein an amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-181 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 102-206 of the amino acid sequence shown in SEQ ID No. 10.

28. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in a fragment corresponding to the amino acid fragment 117-181 of the amino acid sequence of SEQ ID

No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to the amino acid fragment to 98-206 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has been added using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

29. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 4,

Y is the amino acid occupying position 174, 175, 176 or 177 of SEQ ID No. 4,

Z is the amino acid occupying position 98, 99, 100, 101, 102 of SEQ ID No. 10, and

V is the amino acid occupying position 199, 200, 201 or 202 of SEQ ID No. 10.

30. The variant according to claim 28, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-174 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 102-199 of the amino acid sequence shown in SEQ ID No. 10.

31. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to the amino acid fragment 12-19 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment which corresponds to the amino acid fragment 28-42 of SEQ ID No. 10, or in which one or more additional amino acid residues has/have been

inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

32. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 12, 13 or 14 of SEQ ID No. 4,

Y is the amino acid occupying position 15, 16, 17, 18 or 19 of SEQ ID No. 4,

Z is the amino acid occupying position 28, 29, 30, 31 or 32 of SEQ ID No. 10, and

V is an amino acid residue corresponding to the amino acid occupying position 38, 39, 40, 41 or 42 of SEQ ID No. 10.

33. The variant according to claim 31, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 14-15 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 32-38 of the amino acid sequence shown in SEQ ID No. 10.

34. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is present in a fragment corresponding to amino acid residues 7-23 of the amino acid sequence of SEQ ID No. 4, has/have been deleted or replaced with one or more amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 13-45 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

35. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 4, the said region having at least 80%, such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 7 or 8 of SEQ ID No. 4,

Y is the amino acid occupying position 18, 19, 20, 21, 22 or 23 of SEQ ID No. 4,

Z is the amino acid occupying position 13 or 14 of SEQ ID No. 10, and

V is the amino acid occupying position 40, 41, 42, 43, 44 or 45 of SEQ ID No. 10.

36. The variant according to claim 34, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 8-18 of SEQ ID No. 4, has been replaced with the amino acid fragment corresponding to amino acid residues 14-40 of the amino acid sequence shown in SEQ ID No. 10.

37. A variant of a parent Termamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is present in a fragment corresponding to amino acid residues 322-346 of the amino acid sequence of SEQ ID No. 2, has/have been deleted or replaced with one or more amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 291-313 of the amino acid sequence shown in SEQ ID No. 10, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 10 or a corresponding part of another Fungamyl-like α-amylase as a template.

38. A variant of a parent Termamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 2, the said region having at least 80% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 10, wherein

X is the amino acid occupying position 322, 323, 324 or 325 of SEQ ID No. 2,

Y is the amino acid occupying position 343, 344, 345 or 346 of SEQ ID No. 2,

Z is the amino acid occupying position 291, 292, 293 or 294 of SEQ ID No. 10, and

V is the amino acid occupying position 310, 311, 312 or 313 of SEQ ID No. 10.

39. The variant according to claim 37, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 325-345 of SEQ D No. 2, has been replaced with the amino acid fragment corresponding to amino acid residues 294-313 of the amino acid sequence shown in SEQ ID No. 10.

40. A variant of a parent Fungamyl-like α-amylase, in which variant, at least one of the amino acid residues of the parent α-amylase, which is/are present in an amino acid fragment corresponding to amino acid residues 291-313 of the amino acid sequence of SEQ ID No. 10, has/have been deleted or replaced with one or more of the amino acid residues, which is/are present in an amino acid fragment corresponding to amino acid residues 98-210 of the amino acid sequence shown in SEQ ID No. 4, or in which one or more additional amino acid residues has/have been inserted using the relevant part of SEQ ID No. 4 or a corresponding part of another Termamyl-like α-amylase as a template.

41. A variant of a parent Fungamyl-like α-amylase, which variant has a region which, when the amino acid sequence of variant is aligned most closely with the amino acid sequence of the said parent α-amylase, occupies the same position as the portion from residue X to residue Y of SEQ ID No 10, the said region having at least 80%, such as at least 90% sequence homology with the part of SEQ ID No 10 extending from residue Z to residue V of SEQ ID No 4, wherein

X is the amino acid occupying position 117, 118, 119, 120 or 121 of SEQ ID No. 10,

Y is the amino acid occupying position 181, 182, 183, 184 or 185 of SEQ ID No. 10,

Z is the amino acid occupying position 98, 99, 100, 101 or 102 of SEQ ID No. 4, and

V is the amino acid occupying position 206, 207, 208, 209 or 210 of SEQ ID No. 4.

42. The variant according to claim 40, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-181 of SEQ ID No. 10, has been replaced with the amino acid fragment corresponding to amino acid residues 102-206 of the amino acid sequence shown in SEQ ID No. 4.

43. A variant according to claim 40, wherein the amino acid fragment of the parent α-amylase, which corresponds to amino acid residues 121-174 of SEQ ID No. 10, has been replaced with the amino acid fragment corresponding to amino acid residues 102-199 of the amino acid sequence shown in SEQ ID No. 4.

44. A variant of a parent Fungamyl-like α-amylase, wherein an amino acid fragment corresponding to amino acid residues 181-184 of the amino acid sequence shown in SEQ ID No. 10 has been deleted.

45. A variant of a parent Termamyl-like α-amylase, which exhibits α-amylase activity and which has a decreased Ca^{2+} dependency as compared to the parent α-amylase.

46. A variant according to claim 45, which comprises a mutation in a position corresponding to at least one of the following positions in SEQ ID NO 2:

N104, A349, I479, L346, I430, N457, K385, F350, I411, H408 or G303, in particular a mutation corrsponding to

N104D;

A349C+I479C;

L346C+I430C;

N457D,E;

N457D,E+K385R;
F350D,E+I430R,K;
F350D,E+I411R,K;
H408Q,E,N,D; and/or
G303N,D,Q,E.

47. A variant of a parent Termamyl-like α-amylase which exhibits a higher activity below the pH optimum than the parent α-amylase, which variant comprises a mutation of an amino acid residue corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2): E336, Q333, P331, I236, V102, A232, I103, L196, in particular at least one of the following mutations:
E336R,K;
Q333R,K; P331R,K;
V102R,K,A,T,S,G;
I236K,R,N;
I103K,R;
L196K,R; and/or
A232T,S,G.

48. A variant of a parent Termamyl-like α-amylase which exhibits a higher activity above the pH optimum than the parent α-amylase, which variant comprises a mutation of an amino acid residue corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2): N236, H281 and/or Y273, in particular one of the following mutations:
N326I,Y,F,L,V;
H281F,I,L; and/or
Y273F,W.

49. A variant of a parent Termamyl-like α-amylase which exhibits α-amylase activity and which has an increased thermostability and/or altered temperature optimum as compared to the parent α-amylase, which variant comprises a mutation of an amino acid residue

corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2):

L61, Y62, F67, K106, G145, I212, S151, R214, Y150, F143, R146, L241, I236, L7, V259, F284, F350, F343, L427 and/or V481, in particular at least one of the following mutations:

L61W,V,F;

Y62W;

F67W;

K106R,F,W;

G145F,W

I212F,L,W,Y,R,K;

S151 replaced with any other amino acid residue and in particular with F,W,I or L;

R214W;

Y150R,K;

F143W;

R146W;

L241I,F,Y,W;

I236L,F,W,Y;

L7F,I,W;

V259F,I,L;

F284W;

F350W;

F343W;

L427F,L,W; and/or

V481,F,I;L,W.

50. A variant of a parent Termamyl-like α-amylase, which exhibits α-amylase activity and which has a reduced capability of cleaving an oligo-saccharide substrate close to the branching point as compared to the parent α-amylase, which variant comprises a mutation of an amino acid residue corresponding to at least one of the following positions of the *B. licheniformis* α-amylase (SEQ ID NO 2):

V54, D53, Y56, Q333 and/or G57, in particular at least one of the following mutations:

V54L,I,F,Y,W,R,K,H,E,Q;

D53L,I,F,Y,W;

Y56W;

Q333W; and/or

G57 to all possible amino acid residues.

51. The variant according to claim 17, wherein one or more proline residues present in the amino acid residues with which the parent α-amylase is modified are replaced with a non-proline residue such as alanine.

52. The variant according to claim 17, wherein one or more cysteine residues present in the amino acid residues with which the parent α-amylase is modified are replaced with a non-cysteine residue such as alanine.

53. A DNA construct comprising a DNA sequence encoding an α-amylase variant according to claim 17.

54. A recombinant expression vector which carries a DNA construct according to claim 53.

55. A cell which is transformed with a DNA construct according to claim 53.

56. A cell according to claim 55, which is a microorganism.

57. A cell according to claim 56, which is a bacterium or a fungus.

58. The cell according to claim 57, which is a gram positive bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thuringiensis*.

59. Use of an α-amylase variant according to claim 17 for washing and/or dishwashing.

60. Use of an α-amylase variant according to claim 17 for desizing.

61. Use of an α-amylase variant according to claim 17 for starch liquefaction.

62. A detergent additive comprising an α-amylase variant according to claim 17, optionally in the form of a non-dusting granulate, stabilized liquid or protected enzyme.

63. A detergent additive according to claim 62 which contains 0.02-200 mg of enzyme protein/g of the additive.

64. A detergent additive according to claim 62, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

65. A detergent composition comprising an α-amylase variant according to claim 17.

66. A detergent composition according to claim 65 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

67. A manual or automatic dishwashing detergent composition comprising an α-amylase variant according to claim 17.

68. A dishwashing detergent composition according to claim 67 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

69. A manual or automatic laundry washing composition comprising an α-amylase variant according to claim 17.

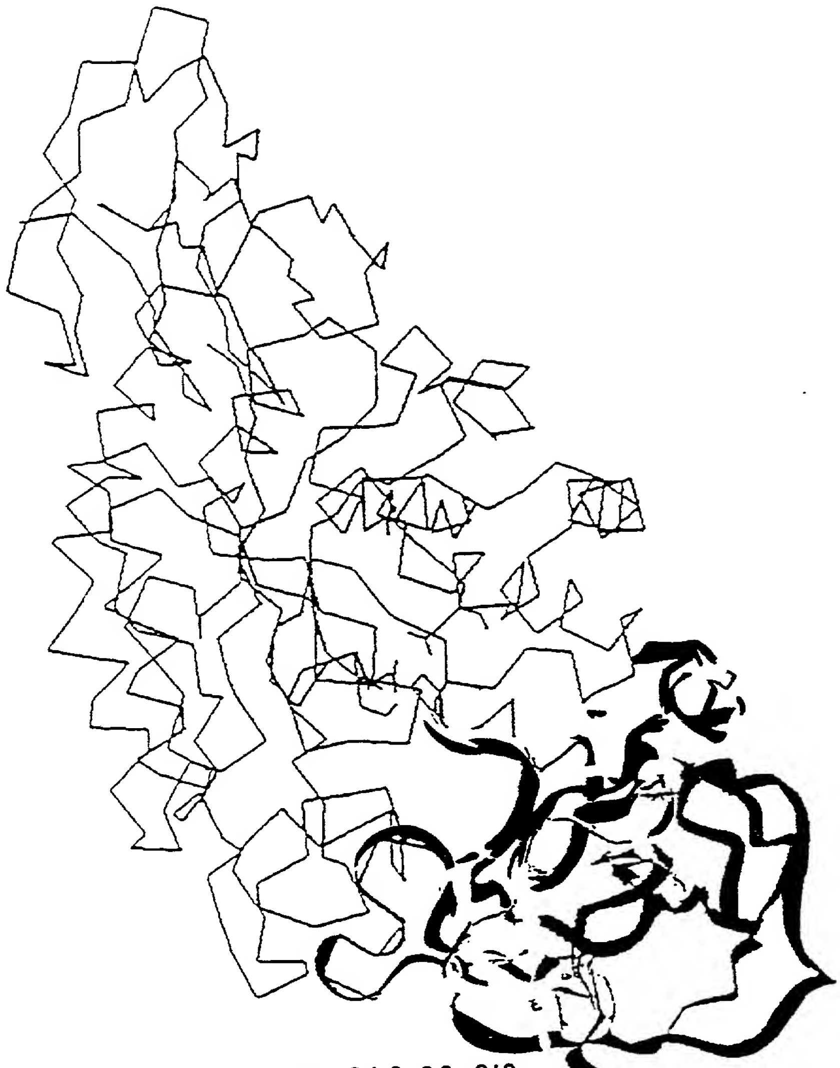
70. A laundry washing composition according to claim 69, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

ABSTRACT

The present invention relates to a method of constructing a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity and at least one altered property as compared to the parent α-amylase, comprising i) analyzing the structure of the parent Termamyl-like α-amylase to identify at least one amino acid residue or at least one structural part of the Termamyl-like α-amylase structure, which amino acid residue or structural part is believed to be of relevance for altering the property of the parent Termamyl-like α-amylase (as evaluated on the basis of structural or functional considerations), ii) constructing a Termamyl-like α-amylase variant, which as compared to the parent Termamyl-like α-amylase, has been modified in the amino acid residue or structural part identified in i) so as to alter the property, and iii) testing the resulting Termamyl-like α-amylase variant for the property in question.

FIG. 1

FIG. 2



LOOP3(b):TAA:117-185,BA2:98-210

FIG. 3

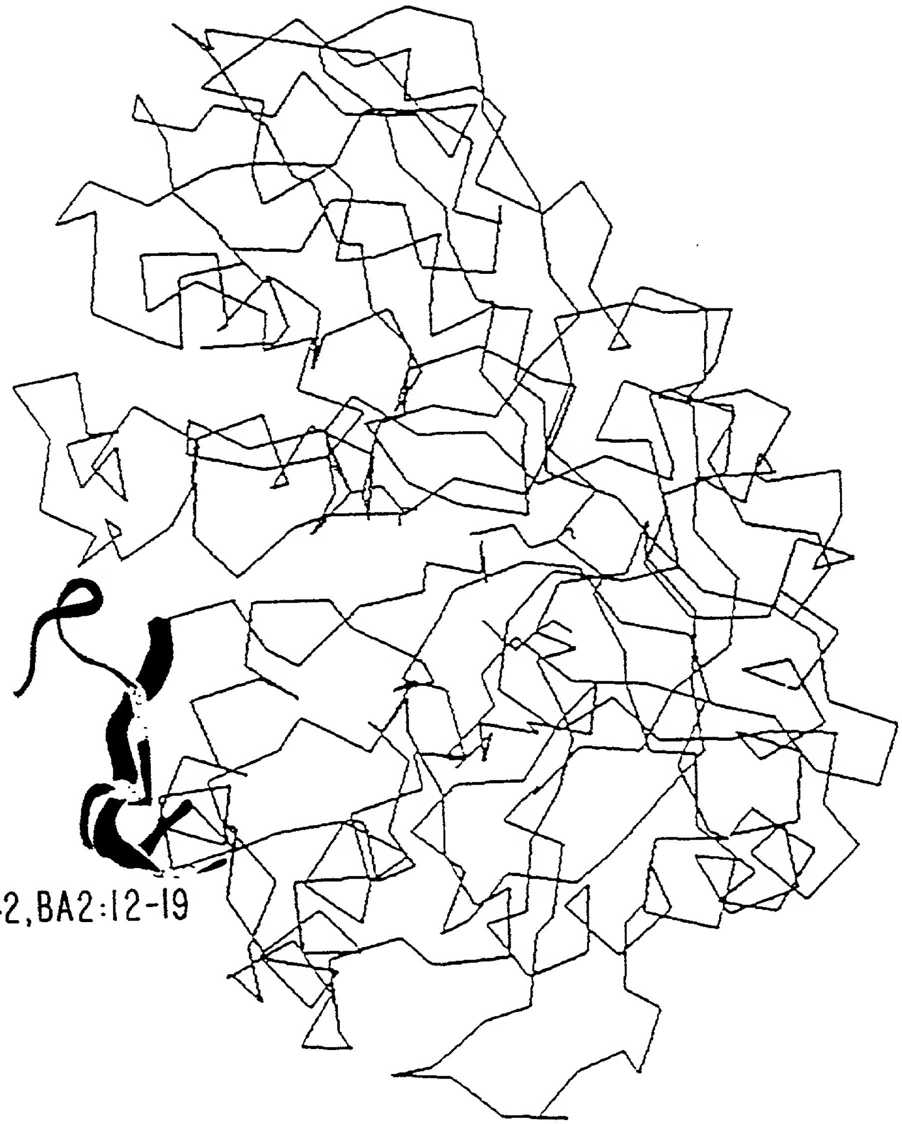


FIG. 4

LOOP1(b):TAA:13-45,A2:7-23

FIG. 5

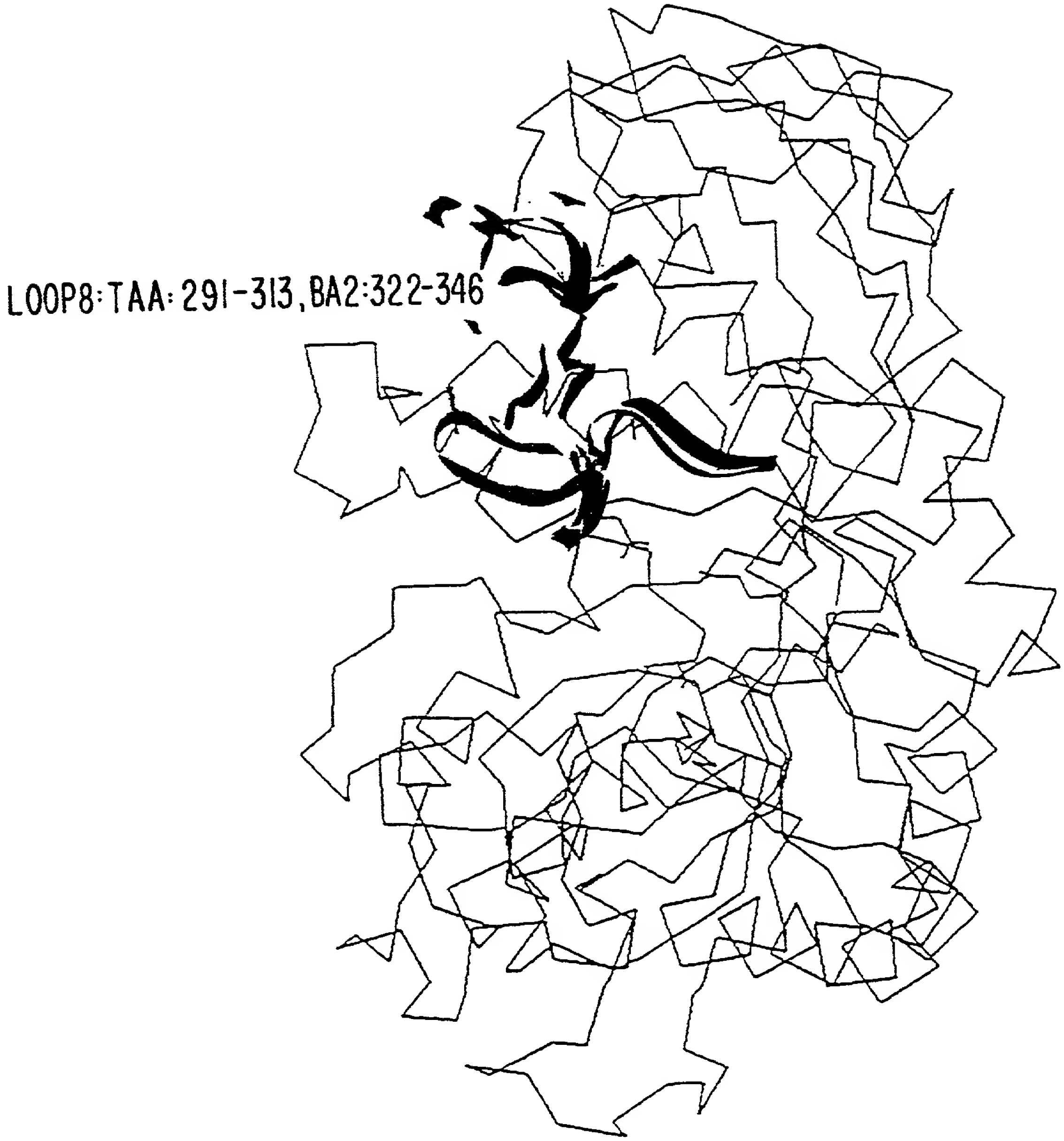


FIG.6

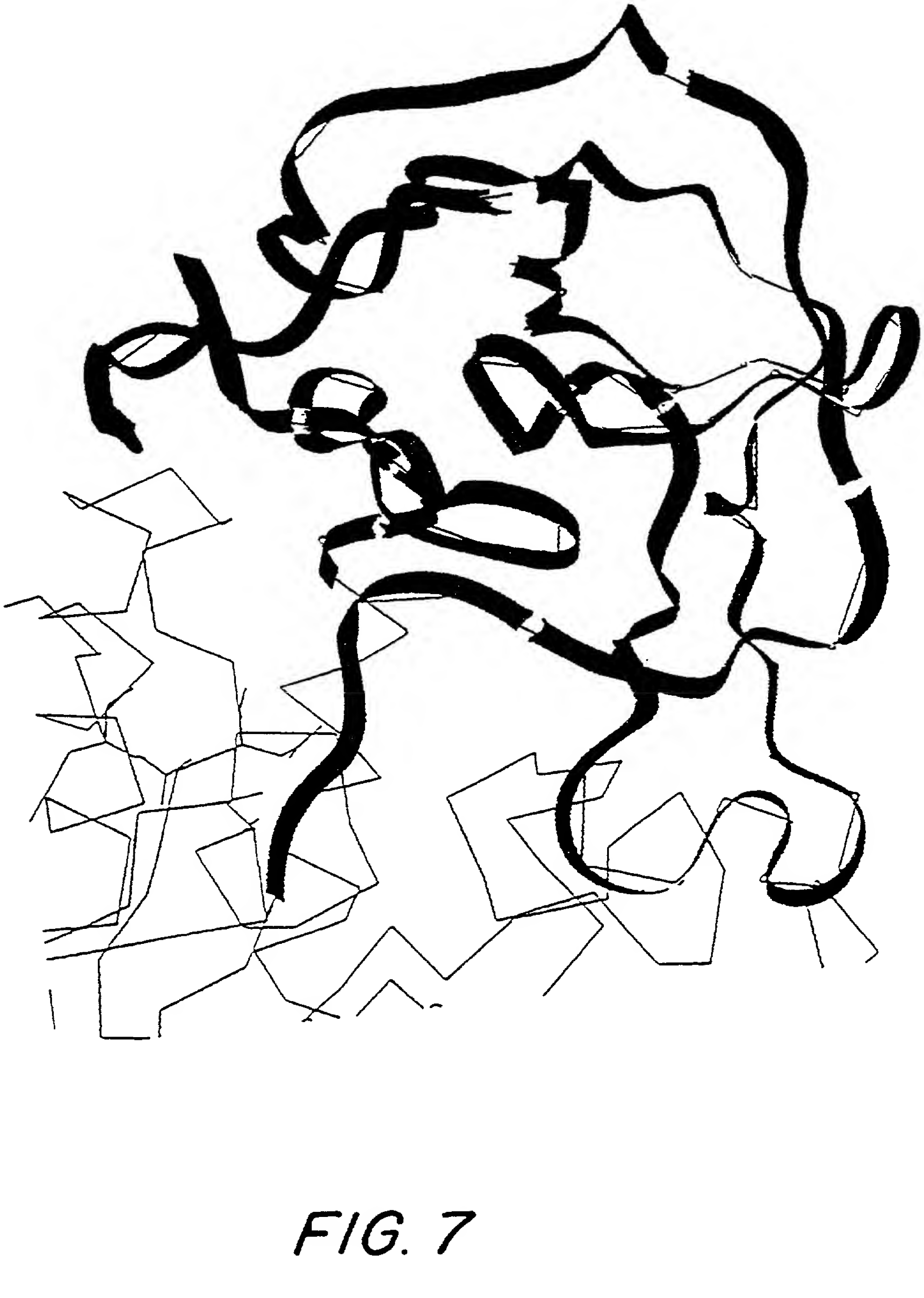


FIG. 7

FIG. 8A

```
1
CAT CAT AAT GGA ACA AAT GGT ACT ATG ATG CAA TAT TTC GAA TGG TAT TTG CCA AAT GAC
H   H   N   G   T   N   G   T   M   M   Q   Y   F   E   W   Y   L   P   N   D

21
GGG AAT CAT TGG AAC AGG TTG AGG GAT GAC GCA GCT AAC TTA AAG AGT AAA GGG ATA ACA
G   N   H   W   N   R   L   R   D   D   A   A   N   L   K   S   K   G   I   T

41
GCT GTA TGG ATC CCA CCT GCA TGG AAG GGG ACT TCC CAG AAT GAT GTA GGT TAT GGA GCC
A   V   W   I   P   P   A   W   K   G   T   S   Q   N   D   V   G   Y   G   A

61
TAT GAT TTA TAT GAT CTT GGA GAG TTT AAC CAG AAG GGG ACG GTT CGT ACA AAA TAT GGA
Y   D   L   Y   D   L   G   E   F   N   Q   K   G   T   V   R   T   K   Y   G

81
ACA CGC AAC CAG CTA CAG GCT GCG GTG ACC TCT TTA AAA AAT AAC GGC ATT CAG GTA TAT
T   R   N   Q   L   Q   A   A   V   T   S   L   K   N   N   G   I   Q   V   Y

101
GGT GAT GTC GTC ATG AAT CAT AAA GGT GGA GCA GAT GGT ACG GAA ATT GTA AAT GCG GTA
G   D   V   V   M   N   H   K   G   G   A   D   G   T   E   I   V   N   A   V
```

FIG.8B

121
GAA GTG AAT CGG AGC AAC CGA AAC CAG GAA ACC TCA GGA GAG TAT GCA ATA GAA GCG TGG
E V N R S N R N Q E T S G E Y A I E A W

141
ACA AAG TTT GAT TTT CCT GGA AGA GGA AAT AAC CAT TCC AGC TTT AAG TGG CGC TGG TAT
T K F D F P G R G N N H S S F K W R W Y

161
CAT TTT GAT GGG ACA GAT TGG GAT CAG TCA CGC CAG CTT CAA AAC AAA ATA TAT AAA TTC
H F D G T D W D Q S R Q L Q N K I Y K F

181
AGG GGA ACA GGC AAG GCC TGG GAC TGG GAA GTC GAT ACA GAG AAT GGC AAC TAT GAC TAT
R G T G K A W D W E V D T E N G N Y D Y

201
CTT ATG TAT GCA GAC GTG GAT ATG GAT CAC CCA GAA GTA ATA CAT GAA CTT AGA AAC TGG
L M Y A D V D M D H P E V I H E L R N W

221
GGA GTG TGG TAT ACG AAT ACA CTG AAC CTT GAT GGA TTT AGA ATA GAT GCA GTG AAA CAT
G V W Y T N T L N L D G F R I D A V K H

FIG. 8C

241
ATA AAA TAT AGC TTT ACG AGA GAT TGG CTT ACA CAT GTG CGT AAC ACC ACA GGT AAA CCA
I K Y S F T R D W L T H V R N T T G K P

261
ATG TTT GCA GTG GCT GAG TTT TGG AAA AAT GAC CTT GGT GCA ATT GAA AAC TAT TTG AAT
M F A V A E F W K N D L G A I E N Y L N

281
AAA ACA AGT TGG AAT CAC TCG GTG TTT GAT GTT CCT CTC CAC TAT AAT TTG TAC AAT GCA
K T S W N H S V F D V P L H Y N L Y N A

301
TCT AAT AGC GGT GGT TAT TAT GAT ATG AGA AAT ATT TTA AAT GGT TCT GTG GTG CAA AAA
S N S G G Y Y D M R N I L N G S V V Q K

321
CAT CCA ACA CAT GCC GTT ACT TTT GTT GAT AAC CAT GAT TCT CAG CCC GGG GAA GCA TTG
H P T H A V T F V D N H D S Q P G E A L

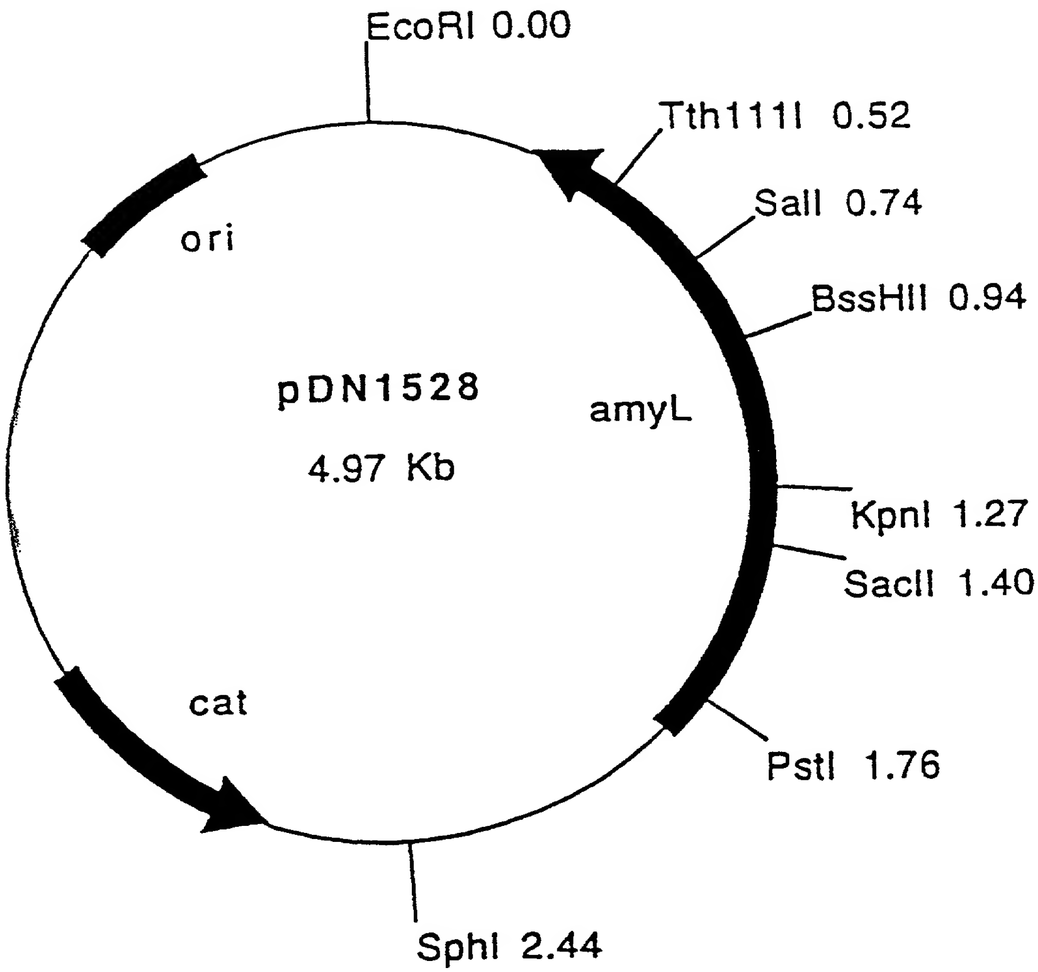
341
GAA TCC TTT GTT CAA CAA TGG TTT AAA CCA CTT GCA TAT GCA TTG GTT CTG ACA AGG GAA
E S F V Q Q W F K P L A Y A L V L T R E

```
361
CAA GGT TAT CCT TCC GTA TTT TAT GGG GAT TAC TAC GGT ATC CCA ACC CAT GGT GTT CCG
Q   G   Y   P   S   V   F   Y   G   D   Y   Y   G   I   P   T   H   G   V   P
381
GCT ATG AAA TCT AAA ATA GAC CCT CTT CTG CAG GCA CGT CAA ACT TTT GCC TAT GGT ACG
A   M   K   S   K   I   D   P   L   L   Q   A   R   Q   T   F   A   Y   G   T
401
CAG CAT GAT TAC TTT GAT CAT CAT GAT ATT ATC GGT TGG ACA AGA GAG GGA AAT AGC TCC
Q   H   D   Y   F   D   H   H   D   I   I   G   W   T   R   E   G   N   S   S
421
CAT CCA AAT TCA GGC CTT GCC ACC ATT ATG TCA GAT GGT CCA GGT GGT AAC AAA TGG ATG
H   P   N   S   G   L   A   T   I   M   S   D   G   P   G   G   N   K   W   M

441
TAT GTG GGG AAA AAT AAA GCG GGA CAA GTT TGG AGA GAT ATT ACC GGA AAT AGG ACA GGC
Y   V   G   K   N   K   A   G   Q   V   W   R   D   I   T   G   N   R   T   G
261
ACC GTC ACA ATT AAT GCA GAC GGA TGG GGT AAT TTC TCT GTT AAT GGA GGG TCC GTT TCG
T   V   T   I   N   A   D   G   W   G   N   F   S   V   N   G   G   S   V   S

481
GTT TGG GTG AAG CAA TAA
V   W   V   K   Q
```

FIG. 8D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KpnI 1.27
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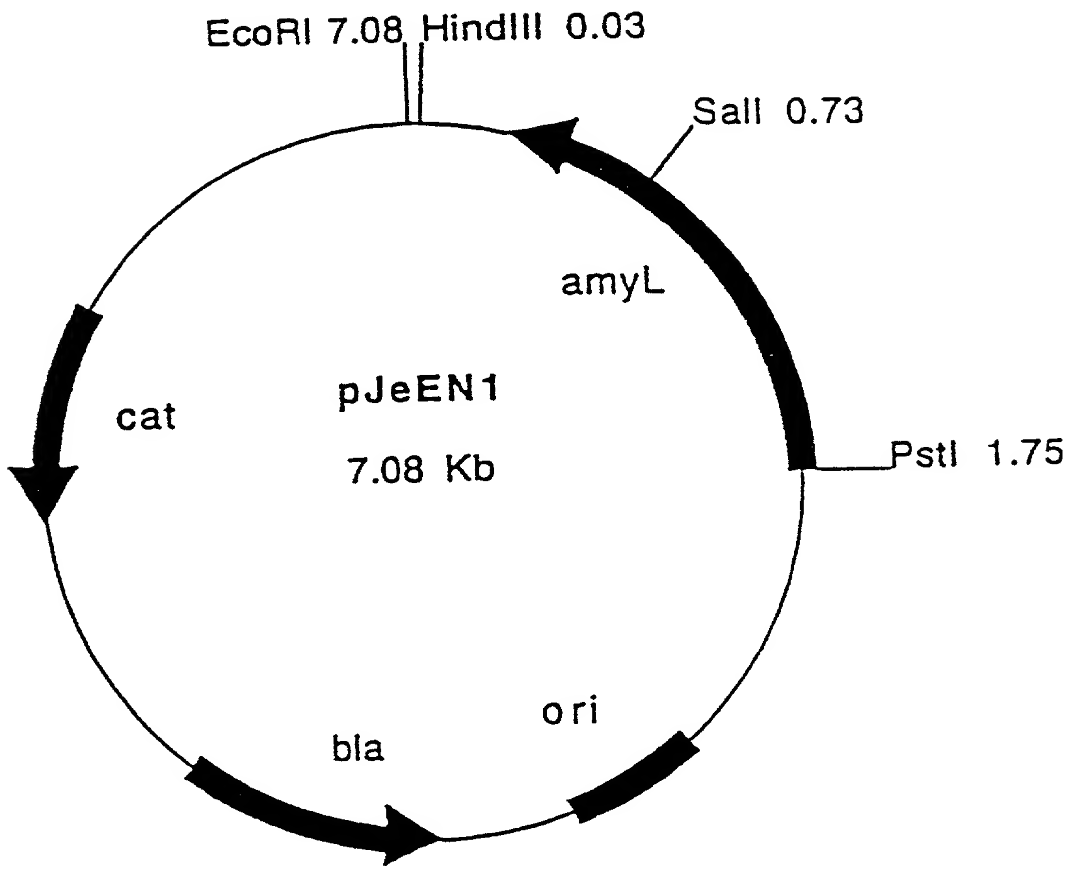
PstI 1.76
SphI 2.44
ori
amyL
cat
pDN1528
4.97 Kb

FIG. 9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HindIII 0.03
SalI 0.73
amyL
pJeEN1
7.08 Kb
cat
PstI 1.75
ori
bla

FIG. 10

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	Attorney's Docket Number. 4394.204-US

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

α-Amylase Mutants

the specification of which (check only one item below):

[ ] is attached hereto

[X] was filed as United States application

Serial No. to be assigned

on February 13, 1996,

and was amended

on

[ ] was filed as PCT international application

Number

on ,

and was amended under PCT Article 19

on

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign applications(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign applications(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Denmark	0128/95	February 3, 1995	[x] YES [ ] NO
Denmark	1192/95	October 23, 1995	[x] YES [ ] NO
Denmark	1256/95	November 10, 1995	[x] YES [ ] NO
			[ ] YES [ ] NO
			[ ] YES [ ] NO
			[ ] YES [ ] NO

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	Attorney's Docket Number 4394.204-US

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this applications is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS			STATUS (Check one)		
U.S APPLICATION NUMBER	U.S. FILING DATE		Patented	Pending	Abandoned
PCT APPLICATIONS DESIGNATING THE U.S.					
APPLICATION NO.	FILING DATE	US SERIAL NUMBER ASSIGNED (if any)			
PCT/DK96/00057	February 5, 1996			x	

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1	Signature of Inventor 2	Signature of Inventor 3
Allan Svendsen		Torben V. Borchert
Date 4/3-1996	Date 29/2-96.	Date 4.3.1996

Attorney Docket No.: 4394.214-US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Svendsen et al.

Serial No.: To be assigned Group Art Unit: To be assigned

Filed: June 3, 1999 Examiner: To be assigned

For: α-Amylase Mutants

REQUEST TRANSFER OF COMPUTER READABLE SEQUENCE LISTING FROM PARENT CASE

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

The computer-readable form in this application is identical with that filed in Application Serial No. 08/600,908 filed February 13, 1996. In accordance with 37 CFR 1.821(e), please use the last filed computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is included in a separately filed preliminary amendment for incorporation into the specification.

The Examiner is hereby to contact the undersigned if there are any questions concerning this response.

Respectfully submitted,

Date: June 3, 1999

Reza Green, Reg. No. 38,475
Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

SEQUENCE LISTING

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Borchert, Torben Vedel

(ii) TITLE OF INVENTION: α-Amylase Mutants

(iii) NUMBER OF SEQUENCES: 13

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(F) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 03-JUN-1999
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Green, Reza
(B) REGISTRATION NUMBER: 38,475
(C) REFERENCE/DOCKET NUMBER: 4394.214-US

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(B) TELEFAX: 212-878-9655

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1920 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 334..1869

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 334..420

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 421..1869

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGATTATTAA AAAGCTGAAA GCAAAAGGCT ATCAATTGGT AACTGTATCT CAGCTTGAAG 180

AAGTGAAGAA GCAGAGAGGC TATTGAATAA ATGAGTAGAA GCGCCATATC GGCGCTTTTC 240

TTTTGGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT TTATACAACA 300

TCATATGTTT CACATTGAAA GGGGAGGAGA ATC ATG AAA CAA CAA AAA CGG CTT 354
Met Lys Gln Gln Lys Arg Leu
-29 -25

TAC GCC CGA TTG CTG ACG CTG TTA TTT GCG CTC ATC TTC TTG CTG CCT 402
Tyr Ala Arg Leu Leu Thr Leu Leu Phe Ala Leu Ile Phe Leu Leu Pro
-20 -15 -10

CAT TCT GCA GCA GCG GCG GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT 450
His Ser Ala Ala Ala Ala Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr
-5 1 5 10

TTT GAA TGG TAC ATG CCC AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA 498
Phe Glu Trp Tyr Met Pro Asn Asp Gly Gln His Trp Arg Arg Leu Gln
15 20 25

AAC GAC TCG GCA TAT TTG GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT 546
Asn Asp Ser Ala Tyr Leu Ala Glu His Gly Ile Thr Ala Val Trp Ile
30 35 40

CCC CCG GCA TAT AAG GGA ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT 594
Pro Pro Ala Tyr Lys Gly Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala
45 50 55

TAC GAC CTT TAT GAT TTA GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG 642
Tyr Asp Leu Tyr Asp Leu Gly Glu Phe His Gln Lys Gly Thr Val Arg
60 65 70

ACA AAG TAC GGC ACA AAA GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT 690
Thr Lys Tyr Gly Thr Lys Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu
75 80 85 90

CAT TCC CGC GAC ATT AAC GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA 738
His Ser Arg Asp Ile Asn Val Tyr Gly Asp Val Val Ile Asn His Lys
95 100 105

GGC GGC GCT GAT GCG ACC GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC 786
Gly Gly Ala Asp Ala Thr Glu Asp Val Thr Ala Val Glu Val Asp Pro
110 115 120

GCT GAC CGC AAC CGC GTA ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG 834
Ala Asp Arg Asn Arg Val Ile Ser Gly Glu His Leu Ile Lys Ala Trp
125 130 135

ACA CAT TTT CAT TTT CCG GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA 882
Thr His Phe His Phe Pro Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys
140 145 150

TGG CAT TGG TAC CAT TTT GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG 930
Trp His Trp Tyr His Phe Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys
155 160 165 170

CTG AAC CGC ATC TAT AAG TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT 978
Leu Asn Arg Ile Tyr Lys Phe Gln Gly Lys Ala Trp Asp Trp Glu Val
175 180 185

TCC AAT GAA AAC GGC AAC TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT 1026
Ser Asn Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp
190 195 200

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TAT GAC CAT CCT GAT GTC GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG       1074
Tyr Asp His Pro Asp Val Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp
        205                 210                 215

TAT GCC AAT GAA CTG CAA TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA       1122
Tyr Ala Asn Glu Leu Gln Leu Asp Gly Phe Arg Leu Asp Ala Val Lys
    220                 225                 230

CAC ATT AAA TTT TCT TTT TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA       1170
His Ile Lys Phe Ser Phe Leu Arg Asp Trp Val Asn His Val Arg Glu
235                 240                 245                 250

AAA ACG GGG AAG GAA ATG TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC       1218
Lys Thr Gly Lys Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp
                255                 260                 265

TTG GGC GCG CTG GAA AAC TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA       1266
Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Phe Asn His Ser
            270                 275                 280

GTG TTT GAC GTG CCG CTT CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG       1314
Val Phe Asp Val Pro Leu His Tyr Gln Phe His Ala Ala Ser Thr Gln
        285                 290                 295

GGA GGC GGC TAT GAT ATG AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC       1362
Gly Gly Gly Tyr Asp Met Arg Lys Leu Leu Asn Gly Thr Val Val Ser
    300                 305                 310

AAG CAT CCG TTG AAA TCG GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG       1410
Lys His Pro Leu Lys Ser Val Thr Phe Val Asp Asn His Asp Thr Gln
315                 320                 325                 330

CCG GGG CAA TCG CTT GAG TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT       1458
Pro Gly Gln Ser Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu
                335                 340                 345

GCT TAC GCT TTT ATT CTC ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC       1506
Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe
            350                 355                 360

TAC GGG GAT ATG TAC GGG ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT       1554
Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro
        365                 370                 375

GCC TTG AAA CAC AAA ATT GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT       1602
Ala Leu Lys His Lys Ile Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr
    380                 385                 390

GCG TAC GGA GCA CAG CAT GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC       1650
Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp His His Asp Ile Val Gly
395                 400                 405                 410

TGG ACA AGG GAA GGC GAC AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA       1698
Trp Thr Arg Glu Gly Asp Ser Ser Val Ala Asn Ser Gly Leu Ala Ala
                415                 420                 425

TTA ATA ACA GAC GGA CCC GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG       1746
Leu Ile Thr Asp Gly Pro Gly Gly Ala Lys Arg Met Tyr Val Gly Arg
            430                 435                 440

CAA AAC GCC GGT GAG ACA TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG       1794
Gln Asn Ala Gly Glu Thr Trp His Asp Ile Thr Gly Asn Arg Ser Glu
        445                 450                 455

CCG GTT GTC ATC AAT TCG GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC       1842
Pro Val Val Ile Asn Ser Glu Gly Trp Gly Glu Phe His Val Asn Gly
    460                 465                 470
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GGG TCG GTT TCA ATT TAT GTT CAA AGA TAGAAGAGCA GAGAGGACGG 1889
Gly Ser Val Ser Ile Tyr Val Gln Arg
475 480

ATTTCCTGAA GGAAATCCGT TTTTTTATTT T 1920

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 512 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
-29 -25 -20 -15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
-10 -5 1

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
5 10 15

Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
20 25 30 35

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
40 45 50

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
55 60 65

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
70 75 80

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
85 90 95

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
100 105 110 115

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
120 125 130

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly
135 140 145

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
150 155 160

Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
165 170 175

Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
180 185 190 195

Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
200 205 210

Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
215 220 225

Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
230 235 240

Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
245 250 255

Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
260 265 270 275

Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
280 285 290

Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
295 300 305

Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
310 315 320

Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
325 330 335

Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
340 345 350 355

Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
360 365 370

Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
375 380 385

Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
390 395 400

Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
405 410 415

Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
420 425 430 435

Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
440 445 450

Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
455 460 465

Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
470 475 480

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2084 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 250..1791

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 250..342

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 343..1791

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GCCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTTGATGACT GATGATTTGG      60

CTGAAGAAGT GGATCGATTG TTTGAGAAAA GAAGAAGACC ATAAAAATAC CTTGTCTGTC     120

ATCAGACAGG GTATTTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAAT AAGGAATAAA     180

GGGGGGTTGT TATTATTTTA CTGATATGTA AAATATAATT TGTATAAGAA AATGAGAGGG     240

AGAGGAAAC ATG ATT CAA AAA CGA AAG CGG ACA GTT TCG TTC AGA CTT         288
          Met Ile Gln Lys Arg Lys Arg Thr Val Ser Phe Arg Leu
          -31 -30                 -25                 -20

GTG CTT ATG TGC ACG CTG TTA TTT GTC AGT TTG CCG ATT ACA AAA ACA       336
Val Leu Met Cys Thr Leu Leu Phe Val Ser Leu Pro Ile Thr Lys Thr
            -15                 -10                 -5

TCA GCC GTA AAT GGC ACG CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG       384
Ser Ala Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro
        1               5                  10

AAC GAC GGC CAG CAT TGG AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA       432
Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu
15                  20                  25                  30

TCG GAT ATC GGA ATC ACT GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA       480
Ser Asp Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
                35                  40                  45

TTG AGC CAA TCC GAT AAC GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA       528
Leu Ser Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu
            50                  55                  60

GGA GAA TTC CAG CAA AAA GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA       576
Gly Glu Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
        65                  70                  75

TCA GAG CTT CAA GAT GCG ATC GGC TCA CTG CAT TCC CGG AAC GTC CAA       624
Ser Glu Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln
    80                  85                  90

GTA TAC GGA GAT GTG GTT TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA       672
Val Tyr Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr
95                  100                 105                 110

GAA GAT GTA ACT GCC GTC GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA       720
Glu Asp Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu
                115                 120                 125

ACT TCG GAG GAA TAT CAA ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG       768
Thr Ser Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro
            130                 135                 140

GGC CGT GGA AAC ACG TAC AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC       816
Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
        145                 150                 155

GAC GGA GCG GAC TGG GAT GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG       864
Asp Gly Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys
    160                 165                 170

TTT CGT GGG GAA GGA AAA GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC       912
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Phe Arg Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn
175 180 185 190

GGC AAC TAT GAC TAT TTA ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT 960
Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro
195 200 205

GAT GTC GTG GCA GAG ACA AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA 1008
Asp Val Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu
210 215 220

CTG TCA TTA GAC GGC TTC CGT ATT GAT GCC GCC AAA CAT ATT AAA TTT 1056
Leu Ser Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe
225 230 235

TCA TTT CTG CGT GAT TGG GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA 1104
Ser Phe Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys
240 245 250

GAA ATG TTT ACG GTT GCG GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC 1152
Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu
255 260 265 270

GAA AAC TAC TTG AAT AAA ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT 1200
Glu Asn Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val
275 280 285

CCG CTT CAT TTC AAT TTA CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT 1248
Pro Leu His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr
290 295 300

GAT ATG AGG CGT TTG CTG GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA 1296
Asp Met Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu
305 310 315

AAG GCG GTT ACA TTT GTT GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA 1344
Lys Ala Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser
320 325 330

TTG GAA TCG ACA GTC CAA ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT 1392
Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe
335 340 345 350

ATT TTG ACA AGA GAA TCC GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG 1440
Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met
355 360 365

TAC GGG ACA AAA GGG ACA TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT 1488
Tyr Gly Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp
370 375 380

AAT ATA GAG CCG ATT TTA AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC 1536
Asn Ile Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro
385 390 395

CAG CAC GAT TAT ATT GAC CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA 1584
Gln His Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu
400 405 410

GGT GAC AGC TCC GCC GCC AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC 1632
Gly Asp Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp
415 420 425 430

GGA CCC GGC GGA TCA AAG CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC 1680
Gly Pro Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly
435 440 445

GAG ACA TGG TAT GAC ATA ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC 1728
Glu Thr Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile
450 455 460

GGA TCT GAC GGC TGG GGA GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC 1776
Gly Ser Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser
465 470 475

ATT TAT GTT CAG AAA TAAGGTAATA AAAAAACACC TCCAAGCTGA GTGCGGGTAT 1831
Ile Tyr Val Gln Lys
480

CAGCTTGGAG GTGCGTTTAT TTTTTCAGCC GTATGACAAG GTCGGCATCA GGTGTGACAA 1891

ATACGGTATG CTGGCTGTCA TAGGTGACAA ATCCGGGTTT TGCGCCGTTT GGCTTTTTCA 1951

CATGTCTGAT TTTTGTATAA TCAACAGGCA CGGAGCCGGA ATCTTTCGCC TTGGAAAAAT 2011

AAGCGGCGAT CGTAGCTGCT TCCAATATGG ATTGTTCATC GGGATCGCTG CTTTTAATCA 2071

CAACGTGGGA TCC 2084

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 514 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ile Gln Lys Arg Lys Arg Thr Val Ser Phe Arg Leu Val Leu Met
-31 -30 -25 -20

Cys Thr Leu Leu Phe Val Ser Leu Pro Ile Thr Lys Thr Ser Ala Val
-15 -10 -5 1

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp Gly
5 10 15

Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp Ile
20 25 30

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser Gln
35 40 45

Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
50 55 60 65

Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu Leu
70 75 80

Gln Asp Ala Ile Gly Ser Lèu His Ser Arg Asn Val Gln Val Tyr Gly
85 90 95

Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp Val
100 105 110

Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser Glu
115 120 125

Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg Gly
130 135 140 145

Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Ala
150 155 160

Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg Gly
165 170 175

Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn Tyr
180 185 190

Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val Val
195 200 205

Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser Leu
210 215 220 225

Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe Leu
230 235 240

Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met Phe
245 250 255

Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn Tyr
260 265 270

Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu His
275 280 285

Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met Arg
290 295 300 305

Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala Val
310 315 320

Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser
325 330 335

Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr
340 345 350

Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr
355 360 365

Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile Glu
370 375 380 385

Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His Asp
390 395 400

Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp Ser
405 410 415

Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly
420 425 430

Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr Trp
435 440 445

Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser Asp
450 455 460 465

Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr Val
470 475 480

Gln Lys

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1814 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 156..1802

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 156..257

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 258..1802

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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AAATTCGATA TTGAAAACGA TTACAAATAA AAATTATAAT AGACGTAAAC GTTCGAGGGT      60

TTGCTCCCTT TTTACTCTTT TTATGCAATC GTTTCCCTTA ATTTTTTGGA AGCCAAACCG     120

TCGAATGTAA CATTTGATTA AGGGGGAAGG GCATT GTG CTA ACG TTT CAC CGC        173
                                       Val Leu Thr Phe His Arg
                                       -34             -30

ATC ATT CGA AAA GGA TGG ATG TTC CTG CTC GCG TTT TTG CTC ACT GTC       221
Ile Ile Arg Lys Gly Trp Met Phe Leu Leu Ala Phe Leu Leu Thr Val
            -25                 -20                 -15

TCG CTG TTC TGC CCA ACA GGA CAG CCC GCC AAG GCT GCC GCA CCG TTT       269
Ser Leu Phe Cys Pro Thr Gly Gln Pro Ala Lys Ala Ala Ala Pro Phe
    -10                 -5                   1

AAC GGC ACC ATG ATG CAG TAT TTT GAA TGG TAC TTG CCG GAT GAT GGC       317
Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu Pro Asp Asp Gly
  5                  10                  15                  20

ACG TTA TGG ACC AAA GTG GCC AAT GAA GCC AAC AAC TTA TCC AGC CTT       365
Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn Leu Ser Ser Leu
                25                  30                  35

GGC ATC ACC GCT CTT TGG CTG CCG CCC GCT TAC AAA GGA ACA AGC CGC       413
Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys Gly Thr Ser Arg
            40                  45                  50

AGC GAC GTA GGG TAC GGA GTA TAC GAC TTG TAT GAC CTC GGC GAA TTC       461
Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
        55                  60                  65

AAT CAA AAA GGG ACC GTC CGC ACA AAA TAC GGA ACA AAA GCT CAA TAT       509
Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ala Gln Tyr
    70                  75                  80

CTT CAA GCC ATT CAA GCC GCC CAC GCC GCT GGA ATG CAA GTG TAC GCC       557
Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met Gln Val Tyr Ala
 85                  90                  95                 100

GAT GTC GTG TTC GAC CAT AAA GGC GGC GCT GAC GGC ACG GAA TGG GTG       605
Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly Thr Glu Trp Val
                105                 110                 115
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GAC GCC GTC GAA GTC AAT CCG TCC GAC CGC AAC CAA GAA ATC TCG GGC 653
Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln Glu Ile Ser Gly
120 125 130

ACC TAT CAA ATC CAA GCA TGG ACG AAA TTT GAT TTT CCC GGG CGG GGC 701
Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe Pro Gly Arg Gly
135 140 145

AAC ACC TAC TCC AGC TTT AAG TGG CGC TGG TAC CAT TTT GAC GGC GTT 749
Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His Phe Asp Gly Val
150 155 160

GAT TGG GAC GAA AGC CGA AAA TTG AGC CGC ATT TAC AAA TTC CGC GGC 797
Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr Lys Phe Arg Gly
165 170 175 180

ATC GGC AAA GCG TGG GAT TGG GAA GTA GAC ACG GAA AAC GGA AAC TAT 845
Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu Asn Gly Asn Tyr
185 190 195

GAC TAC TTA ATG TAT GCC GAC CTT GAT ATG GAT CAT CCC GAA GTC GTG 893
Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His Pro Glu Val Val
200 205 210

ACC GAG CTG AAA AAC TGG GGG AAA TGG TAT GTC AAC ACA ACG AAC ATT 941
Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn Thr Thr Asn Ile
215 220 225

GAT GGG TTC CGG CTT GAT GCC GTC AAG CAT ATT AAG TTC AGT TTT TTT 989
Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Phe
230 235 240

CCT GAT TGG TTG TCG TAT GTG CGT TCT CAG ACT GGC AAG CCG CTA TTT 1037
Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly Lys Pro Leu Phe
245 250 255 260

ACC GTC GGG GAA TAT TGG AGC TAT GAC ATC AAC AAG TTG CAC AAT TAC 1085
Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys Leu His Asn Tyr
265 270 275

ATT ACG AAA ACA GAC GGA ACG ATG TCT TTG TTT GAT GCC CCG TTA CAC 1133
Ile Thr Lys Thr Asp Gly Thr Met Ser Leu Phe Asp Ala Pro Leu His
280 285 290

AAC AAA TTT TAT ACC GCT TCC AAA TCA GGG GGC GCA TTT GAT ATG CGC 1181
Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala Phe Asp Met Arg
295 300 305

ACG TTA ATG ACC AAT ACT CTC ATG AAA GAT CAA CCG ACA TTG GCC GTC 1229
Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro Thr Leu Ala Val
310 315 320

ACC TTC GTT GAT AAT CAT GAC ACC GAA CCC GGC CAA GCG CTG CAG TCA 1277
Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln Ala Leu Gln Ser
325 330 335 340

TGG GTC GAC CCA TGG TTC AAA CCG TTG GCT TAC GCC TTT ATT CTA ACT 1325
Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr
345 350 355

CGG CAG GAA GGA TAC CCG TGC GTC TTT TAT GGT GAC TAT TAT GGC ATT 1373
Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp Tyr Tyr Gly Ile
360 365 370

CCA CAA TAT AAC ATT CCT TCG CTG AAA AGC AAA ATC GAT CCG CTC CTC 1421
Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile Asp Pro Leu Leu
375 380 385

ATC GCG CGC AGG GAT TAT GCT TAC GGA ACG CAA CAT GAT TAT CTT GAT 1469
Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His Asp Tyr Leu Asp
390 395 400

CAC TCC GAC ATC ATC GGG TGG ACA AGG GAA GGG GGC ACT GAA AAA CCA 1517
His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Gly Thr Glu Lys Pro
405 410 415 420

GGA TCC GGA CTG GCC GCA CTG ATC ACC GAT GGG CCG GGA GGA AGC AAA 1565
Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ser Lys
425 430 435

TGG ATG TAC GTT GGC AAA CAA CAC GCT GGA AAA GTG TTC TAT GAC CTT 1613
Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val Phe Tyr Asp Leu
440 445 450

ACC GGC AAC CGG AGT GAC ACC GTC ACC ATC AAC AGT GAT GGA TGG GGG 1661
Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser Asp Gly Trp Gly
455 460 465

GAA TTC AAA GTC AAT GGC GGT TCG GTT TCG GTT TGG GTT CCT AGA AAA 1709
Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp Val Pro Arg Lys
470 475 480

ACG ACC GTT TCT ACC ATC GCT CGG CCG ATC ACA ACC CGA CCG TGG ACT 1757
Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr Arg Pro Trp Thr
485 490 495 500

GGT GAA TTC GTC CGT TGG ACC GAA CCA CGG TTG GTG GCA TGG CCT 1802
Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val Ala Trp Pro
505 510 515

TGATGCCTGC GA 1814

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 549 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Leu Thr Phe His Arg Ile Ile Arg Lys Gly Trp Met Phe Leu Leu
-34 -30 -25 -20

Ala Phe Leu Leu Thr Val Ser Leu Phe Cys Pro Thr Gly Gln Pro Ala
-15 -10 -5

Lys Ala Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp
1 5 10

Tyr Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala
15 20 25 30

Asn Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala
35 40 45

Tyr Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu
50 55 60

Tyr Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr
65 70 75

Gly Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala
80 85 90

Gly Met Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala
95 100 105 110

Asp Gly Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg
115 120 125

Asn Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe
130 135 140

Asp Phe Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp
145 150 155

Tyr His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg
160 165 170

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
175 180 185 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met
195 200 205

Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
210 215 220

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His
225 230 235

Ile Lys Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln
240 245 250

Thr Gly Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile
255 260 265 270

Asn Lys Leu His Asn Tyr Ile Thr Lys Thr Asp Gly Thr Met Ser Leu
275 280 285

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
290 295 300

Gly Ala Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp
305 310 315

Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro
320 325 330

Gly Gln Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala
335 340 345 350

Tyr Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser
370 375 380

Lys Ile Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr
385 390 395

Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu
400 405 410

Gly Gly Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp
415 420 425 430

Gly Pro Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly
435 440 445

Lys Val Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile
450 455 460

Asn Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser
465 470 475

Val Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile
480 485 490

Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg
495 500 505 510

Leu Val Ala Trp Pro
515

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGTCGTAGGC ACCGTAGCCC CAATCCGCTT G 31

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTCGTAGGC ACCGTAGCCC CAATCCCATT GGCTCG 36

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTGACTGG TGAGTACTCA ACCAAGTC 28

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 478 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr Phe Leu Leu Thr
1               5                   10                  15

Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Thr Cys Asn Thr
            20                  25                  30

Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly Ile Ile Asp Lys
        35                  40                  45

Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile Trp Ile Thr Pro
    50                  55                  60

Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly Asp Ala Tyr His
65                  70                  75                  80

Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu Asn Tyr Gly Thr
                85                  90                  95

Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His Glu Arg Gly Met
            100                 105                 110

Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly Tyr Asp Gly Ala
        115                 120                 125

Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe Ser Ser Gln Asp
    130                 135                 140

Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu Asp Gln Thr Gln
145                 150                 155                 160

Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser Leu Pro Asp Leu
                165                 170                 175

Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr Asp Trp Val Gly
            180                 185                 190

Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg Ile Asp Thr Val
        195                 200                 205

Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn Lys Ala Ala Gly
    210                 215                 220

Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro Ala Tyr Thr Cys
225                 230                 235                 240

Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr Pro Ile Tyr Tyr
                245                 250                 255

Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser Met Asp Asp Leu
            260                 265                 270

Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro Asp Ser Thr Leu
        275                 280                 285
```

Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg Phe Ala Ser Tyr
290 295 300

Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala Phe Ile Ile Leu
305 310 315 320

Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu Gln His Tyr Ala
325 330 335

Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp Leu Ser Gly Tyr
340 345 350

Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser Ala Asn Ala Ile
355 360 365

Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val Thr Tyr Lys Asn
370 375 380

Trp Pro Ile Tyr Lys Asp Asp Ile Thr Ile Ala Met Arg Lys Gly Thr
385 390 395 400

Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys Gly Ala Ser Gly
405 410 415

Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr Thr Ala Gly Gln
420 425 430

Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr Val Gly Ser Asp
435 440 445

Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro Arg Val Leu Tyr
450 455 460

Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser Ser Ser
465 470 475

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1458 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1455

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAT CAT AAT GGA ACA AAT GGT ACT ATG ATG CAA TAT TTC GAA TGG TAT 48
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
520 525 530

TTG CCA AAT GAC GGG AAT CAT TGG AAC AGG TTG AGG GAT GAC GCA GCT 96
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
535 540 545

AAC TTA AAG AGT AAA GGG ATA ACA GCT GTA TGG ATC CCA CCT GCA TGG 144
Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
550 555 560

AAG GGG ACT TCC CAG AAT GAT GTA GGT TAT GGA GCC TAT GAT TTA TAT 192
Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr

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    565                 570                 575

GAT CTT GGA GAG TTT AAC CAG AAG GGG ACG GTT CGT ACA AAA TAT GGA          240
Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
580                 585                 590                 595

ACA CGC AAC CAG CTA CAG GCT GCG GTG ACC TCT TTA AAA AAT AAC GGC          288
Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
                600                 605                 610

ATT CAG GTA TAT GGT GAT GTC GTC ATG AAT CAT AAA GGT GGA GCA GAT          336
Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            615                 620                 625

GGT ACG GAA ATT GTA AAT GCG GTA GAA GTG AAT CGG AGC AAC CGA AAC          384
Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
        630                 635                 640

CAG GAA ACC TCA GGA GAG TAT GCA ATA GAA GCG TGG ACA AAG TTT GAT          432
Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
    645                 650                 655

TTT CCT GGA AGA GGA AAT AAC CAT TCC AGC TTT AAG TGG CGC TGG TAT          480
Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
660                 665                 670                 675

CAT TTT GAT GGG ACA GAT TGG GAT CAG TCA CGC CAG CTT CAA AAC AAA          528
His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
                680                 685                 690

ATA TAT AAA TTC AGG GGA ACA GGC AAG GCC TGG GAC TGG GAA GTC GAT          576
Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
            695                 700                 705

ACA GAG AAT GGC AAC TAT GAC TAT CTT ATG TAT GCA GAC GTG GAT ATG          624
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
        710                 715                 720

GAT CAC CCA GAA GTA ATA CAT GAA CTT AGA AAC TGG GGA GTG TGG TAT          672
Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
    725                 730                 735

ACG AAT ACA CTG AAC CTT GAT GGA TTT AGA ATA GAT GCA GTG AAA CAT          720
Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
740                 745                 750                 755

ATA AAA TAT AGC TTT ACG AGA GAT TGG CTT ACA CAT GTG CGT AAC ACC          768
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
                760                 765                 770

ACA GGT AAA CCA ATG TTT GCA GTG GCT GAG TTT TGG AAA AAT GAC CTT          816
Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            775                 780                 785

GGT GCA ATT GAA AAC TAT TTG AAT AAA ACA AGT TGG AAT CAC TCG GTG          864
Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
        790                 795                 800

TTT GAT GTT CCT CTC CAC TAT AAT TTG TAC AAT GCA TCT AAT AGC GGT          912
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
    805                 810                 815

GGT TAT TAT GAT ATG AGA AAT ATT TTA AAT GGT TCT GTG GTG CAA AAA          960
Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
820                 825                 830                 835

CAT CCA ACA CAT GCC GTT ACT TTT GTT GAT AAC CAT GAT TCT CAG CCC         1008
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His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                840                 845                 850

GGG GAA GCA TTG GAA TCC TTT GTT CAA CAA TGG TTT AAA CCA CTT GCA       1056
Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
            855                 860                 865

TAT GCA TTG GTT CTG ACA AGG GAA CAA GGT TAT CCT TCC GTA TTT TAT       1104
Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        870                 875                 880

GGG GAT TAC TAC GGT ATC CCA ACC CAT GGT GTT CCG GCT ATG AAA TCT       1152
Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
    885                 890                 895

AAA ATA GAC CCT CTT CTG CAG GCA CGT CAA ACT TTT GCC TAT GGT ACG       1200
Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
900                 905                 910                 915

CAG CAT GAT TAC TTT GAT CAT CAT GAT ATT ATC GGT TGG ACA AGA GAG       1248
Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
                920                 925                 930

GGA AAT AGC TCC CAT CCA AAT TCA GGC CTT GCC ACC ATT ATG TCA GAT       1296
Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
            935                 940                 945

GGT CCA GGT GGT AAC AAA TGG ATG TAT GTG GGG AAA AAT AAA GCG GGA       1344
Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
        950                 955                 960

CAA GTT TGG AGA GAT ATT ACC GGA AAT AGG ACA GGC ACC GTC ACA ATT       1392
Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
    965                 970                 975

AAT GCA GAC GGA TGG GGT AAT TTC TCT GTT AAT GGA GGG TCC GTT TCG       1440
Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
980                 985                 990                 995

GTT TGG GTG AAG CAA TAA                                               1458
Val Trp Val Lys Gln
                1000
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
  1               5                  10                  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
            20                  25                  30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60
```

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Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65                  70                  75                  80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
                 85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
        115                 120                 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
        195                 200                 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
                245                 250                 255

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
    290                 295                 300

Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
305                 310                 315                 320

His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335

Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
    370                 375                 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415
```

Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
435 440 445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Val Trp Val Lys Gln
485

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 483 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
1 5 10 15
Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
20 25 30
Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser
35 40 45
Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu
50 55 60
Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
65 70 75 80
Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr
85 90 95
Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
100 105 110
Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser
115 120 125
Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg
130 135 140
Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly
145 150 155 160
Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg
165 170 175
Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn
180 185 190
Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val
195 200 205
Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser
210 215 220
Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe
225 230 235 240
Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met
245 250 255
Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn
260 265 270
Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
275 280 285
His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
290 295 300
Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305 310 315 320

```
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335
Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350
Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365
Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
    370                 375                 380
Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385                 390                 395                 400
Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415
Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430
Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
        435                 440                 445
Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
    450                 455                 460
Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465                 470                 475                 480
Val Gln Arg
```